

AD \_\_\_\_\_

GRANT NUMBER DAMD17-94-J-4347

TITLE: Engineering Bispecific Antibodies that Target ErbB-2 on  
Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Dr. David M. Krantz

CONTRACTING ORGANIZATION: University of Illinois  
Urbana, Illinois 61801

REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander  
U.S. Army Medical Research and Materiel Command  
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1996	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 95 - 31 Aug 96)	
4. TITLE AND SUBTITLE Engineering Bispecific Antibodies that Target ErbB-2 on Breast Cancer Cells			5. FUNDING NUMBERS DAMD17-94-J-4347	
6. AUTHOR(S) Dr. David M. Krantz				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Illinois Urbana, Illinois 61801			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, MD 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19961127 053	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200)  The goals of this project are to construct single-chain bispecific antibodies (scFv <sub>2</sub> ) that target erbB-2 on breast cancer cells, to evaluate the susceptibility of the tumor cells to lysis mediated by bispecific antibodies, and to develop an animal model that can evaluate the <i>in vivo</i> effectiveness of these agents. The following aims were accomplished during the past year: 1) Two different scFv <sub>2</sub> were constructed, purified, and tested <i>in vitro</i> . Both antibodies contained the anti-erbB-2 scFv, 800E6. One of the antibodies contained the anti-T cell receptor scFv 1B2 and the other contained the anti-Vβ scFv KJ16. Although both scFv <sub>2</sub> bound to the appropriate antigens, current efforts are devoted to increasing the fraction of the scFv <sub>2</sub> that have activity. 2) Transgenic mice (called TCR/RAG <sup>-/-</sup> ) that contain a single population of cytotoxic T cells were fully characterized. The mice expressed >90% CD8+ peripheral T cells, which could be activated <i>in vitro</i> to lyse the appropriate target cells. The TCR/RAG <sup>-/-</sup> mice also accepted xenografts of two erbB-2 <sup>+</sup> human tumor cell lines, BT474 and SKOV3. Future work will involve strategies to activate T cells <i>in vivo</i> , so that the optimal conditions for tumor targeting can be explored.				
14. SUBJECT TERMS Bispecific, Antibodies, Immunotherapy, Cytotoxicity, Animal, Model, ErbB-2, Breast Cancer			15. NUMBER OF PAGES 109	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

\_\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

DMK In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

\_\_\_\_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

DMK In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

DMK In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

\_\_\_\_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

David M. Kravitz 9/1/96  
PI - Signature Date

## TABLE OF CONTENTS

FRONT COVER.....	1
REPORT DOCUMENTATION PAGE.....	2
FOREWORD .....	3
TABLE OF CONTENTS.....	4
INTRODUCTION.....	4
BODY.....	5
Specific Aim 1.....	5
Specific Aim 3.....	10
CONCLUSIONS.....	13
Work Completed.....	13
Publications in the Past Year.....	14
Future Work.....	15
REFERENCES.....	15
APPENDIX (three manuscripts, <i>submitted</i> ).....	18

### INTRODUCTION (Adapted in part from original proposal)

Successful treatment of breast cancer requires the identification of specific targets for the rational design of therapeutic agents. One such target, described several years ago, is the oncogene product erbB-2 (1-3). This protein is expressed on the surface of tumor cells in approximately 30% of women with the poorest prognosis for survival (4). The expression of erbB-2 does not appear to be correlated, either positively or negatively, with the estrogen receptor (5), although a recent report suggests that the erbB-2 signalling pathway targets the estrogen receptor resulting in estrogen-independent growth of tumor cells (6). However, its expression is associated with an increase in the resistance of tumor cells to lysis by some natural immune mechanisms such as tumor necrosis factor and lymphokine activated killer cells (7,8).

A number of investigators have now begun to use antibodies specific for erbB-2 as possible therapeutic agents (9-16). The agents include immunotoxins, radioimmunoconjugates, and bispecific antibodies. The latter antibodies are intended to mediate effects by directing the lysis of tumor cells through cytotoxic effector cells. One of the predicted advantages of bispecific antibodies is that they should not have side effects associated with delivery of toxins or isotopes. Bispecific antibodies have in fact begun to be examined in several clinical trials, with some encouraging results.

Despite their promise and emergence into clinical trials, there are many questions that need to be addressed before optimal uses, with minimal side effects, of bispecific antibodies can be realized. For example, genetic engineering now provides a method for constructing smaller, potentially more stable, antibodies. Are these antibodies likely to be more effective than conventional intact antibodies? Will the natural resistance of erbB-2<sup>+</sup> tumors cells present an obstacle to successful bispecific antibody therapy? Can a system that uses a patients own immune cells be developed so that *ex vivo* activation of effector cells is not needed? This would obviously allow the treatment of a much larger patient base than would be possible if effector cells must be cultured for every patient.

It would clearly be useful to have a system that could provide answers to these questions in order to design the most effective clinical trials of bispecific antibodies. There has been no animal model developed that can evaluate all of these issues using human breast cancer cells. The purpose of this project is twofold. First, to construct novel bispecific antibodies that can be expressed as a single-chain in *E. coli* (17). Second, to develop an *in vivo* animal model that will allow the testing of these agents in comparison with other conventional bispecific antibodies. There are many potential therapeutic regimens that will need to be evaluated. To do so, an animal model that will not require introduction of human effector cells and that will most resemble the situation that will be encountered in the human disease will be developed. It is anticipated that these animals could also serve as models for immune modulating agents that are developed by other laboratories.

The methods used to approach these goals involve many reagents and strains of mice that have been developed in my lab and in collaboration with others over the past ten years. The general strategy involves the use of the following: 1) Monoclonal antibodies and single-chain antibodies that are specific for the T cell receptor of a mouse cytotoxic T lymphocyte clone 2C (18); 2) Monoclonal antibodies that are specific for human erbB-2, provided by Pier Natali (11); 3) Cloning and expression systems for the overproduction of single-chain monospecific and bispecific antibodies (17,18); 4) Human breast cancer tumor cell lines obtained from the ATCC; 5) Transgenic mice that express the TCR from CTL clone 2C (19,20); and 6) RAG-1 knockout mice, provided by Susumu Tonegawa's lab, that lack B and T cells because of the absence of recombinational-activation-gene 1 (21).

The four specific aims of the project are: 1) Following a strategy that has recently proven successful in our lab, to engineer a single-chain bispecific antibody to erbB-2 and the TCR of a mouse CTL clone; 2) To use a simple screening method to search for agents that increase the sensitivity of erbB-2<sup>+</sup> breast cancer cell lines to lysis by CTL. 3) To develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, TCR transgenic mice (TCR/recombination-activating-gene knockouts, RAG-2<sup>-</sup>). 4) To test the *in vivo* effectiveness of various bispecific antibody regimens in the TCR/RAG-2<sup>-</sup> human xenograft system. Specific details concerning progress in the second year of this award toward these goals are provided in the body of this report, below.

### BODY

Additional progress has been made toward various aspects of specific aims 1 and 3. Methods, results, and a brief discussion are presented within each aim:

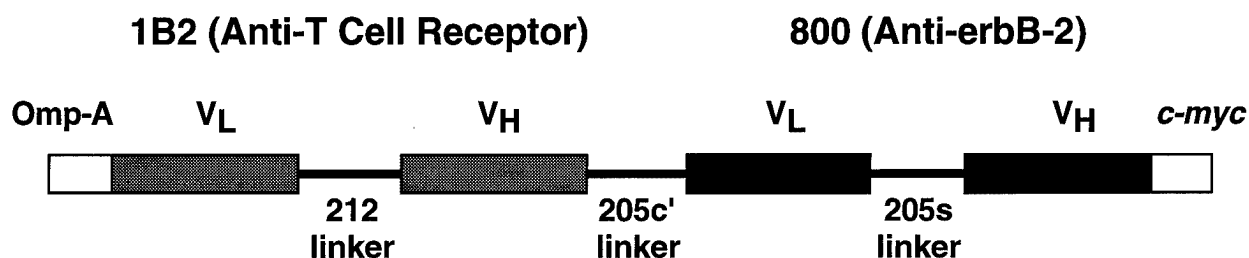
#### **Specific Aim 1. Construct and characterize a single-chain bispecific antibody (anti-erbB-2/anti-TCR, called scFv<sub>2</sub>).**

In last year's report, the cloning, expression, and characterization of the V<sub>H</sub> and V<sub>L</sub> genes from the anti-erbB-2 antibody 800E6 was described. This single-chain gene has now been cloned downstream of the anti-TCR antibodies 1B2 and KJ16 to produce two different scFv<sub>2</sub>. 1B2 recognizes a clonotypic determinant on the TCR from CTL clone 2C (22) while KJ16 recognizes the V $\beta$ 8 chain of clone 2C (23). Both scFv<sub>2</sub> contained the 25 residue interchain linker 205 and a 10 residue *c-myc* tail for detection (Figure 1). These two antibodies were constructed in order to evaluate the effect of different antibody domains on folding of the scFv<sub>2</sub> and ultimately on their bispecific activity.

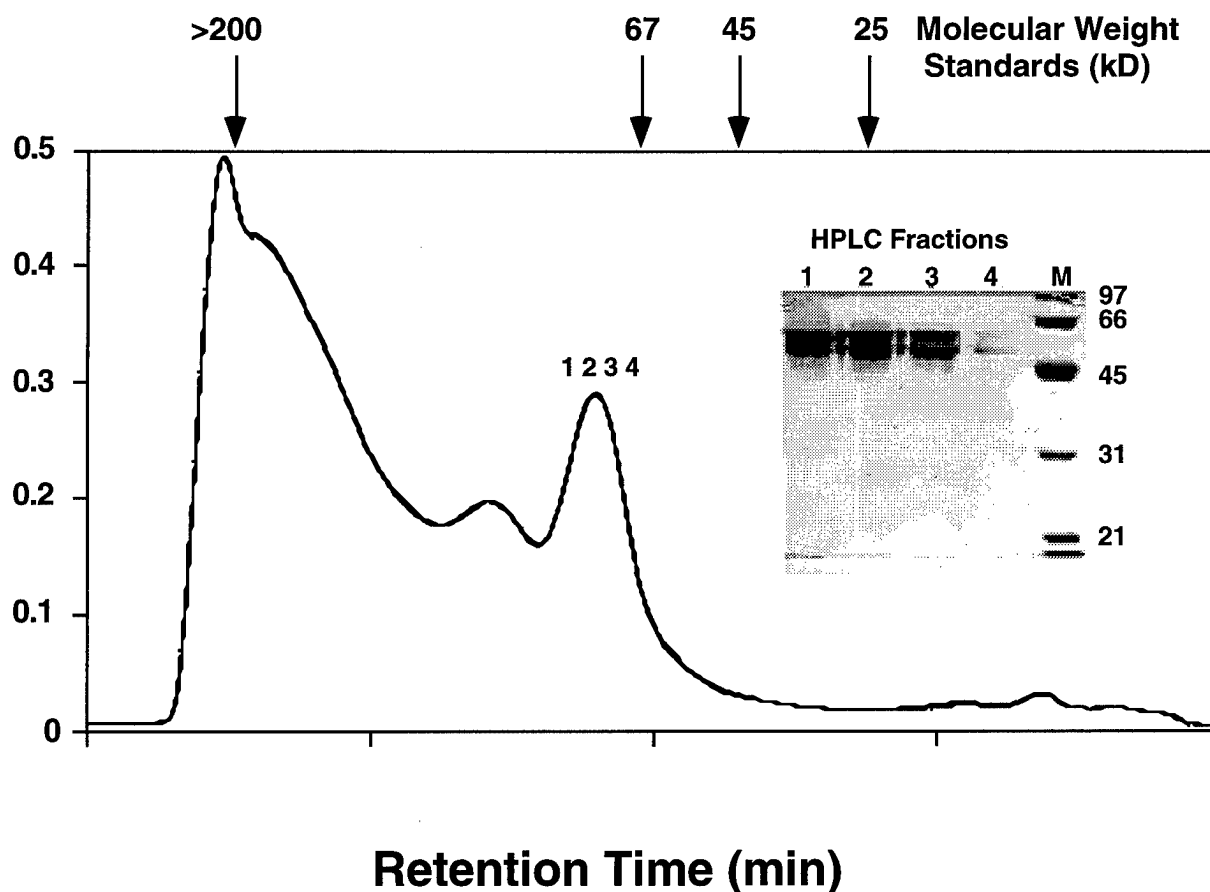
**Construction and Expression of scFv<sub>2</sub>.** To construct the scFv<sub>2</sub>, the 800E6 scFv was amplified by PCR using a 5' primer which contained the sequence for the 205c' linker, and a 3' primer that included a HindIII site within the *c-myc* region for cloning into the 1B2 scFv vector or the KJ16 scFv vector. The linker/800E6 scFv fragment was cloned between the 1B2V<sub>H</sub> (or KJ16 V<sub>H</sub>) and the *c-myc* tail. Inclusion body pellets from cells that contained the scFv<sub>2</sub> genes were solubilized in 6M guanidine. To isolate monomeric scFv<sub>2</sub> from proteins which remained aggregated in the presence of guanidine, the solubilized inclusion body was subjected to Superdex

G-200 size exclusion in 4M guanidine. The 60 kDa peak fractions were individually refolded by dialysis into Tris-Arginine buffer, and analyzed by SDS-PAGE (data not shown). Fractions with the highest fraction of homogeneous scFv2 were pooled, dialysed against buffer and monomeric scFv2 was isolated by non-denaturing size exclusion chromatography (Figure 2). The peak that represented monomer had a 60kD apparent molecular weight under reducing and non-reducing conditions (data not shown). As expected, this was approximately twice the size of either the 1B2, KJ16, or 800E6 scFv (30kD, Figure 2).

**Figure 1. Schematic of scFv<sub>2</sub> gene for single-chain bispecific anti-TCR/anti-erbB-2 antibodies.**

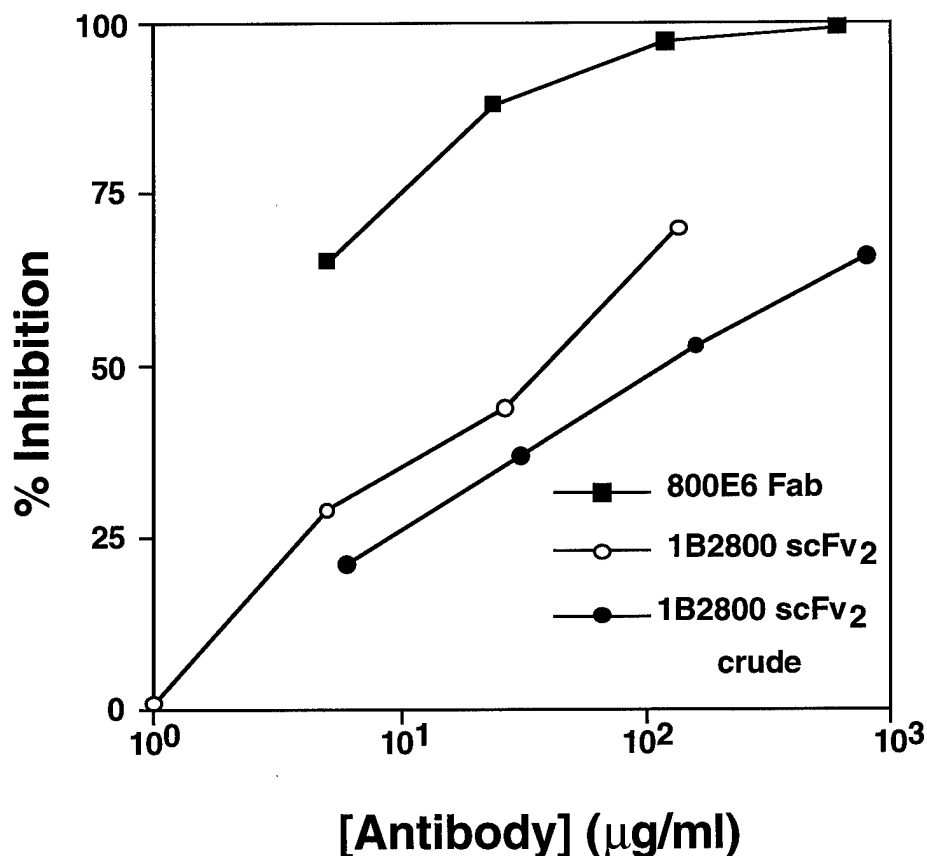


**Figure 2. Gel filtration (G200) profile and SDS-PAGE analysis of 1B2/800E6 scFv<sub>2</sub> protein.**



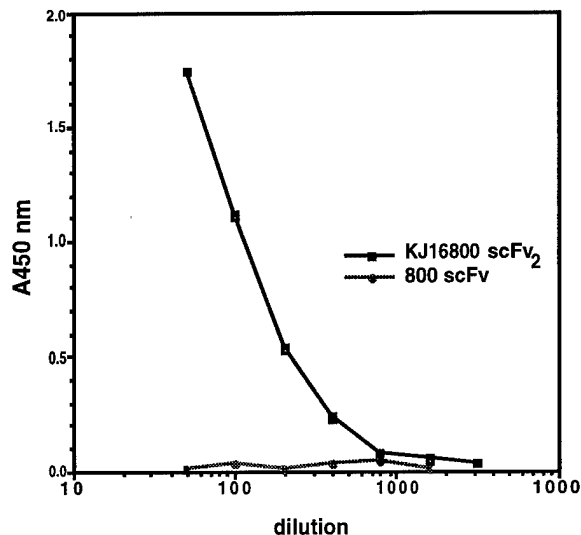
**Binding Properties of Anti-TCR/erbB-2 scFv<sub>2</sub> Antibodies.** To examine binding of the scFv<sub>2</sub> to erbB-2 on the surface of human breast cancer cells, a competition assay was performed with <sup>125</sup>I-labeled 800E6 Fab fragments. As shown in Figure 3, crude as well as partially purified preparations of 1B2 scFv<sub>2</sub> were able to bind erbB-2 on SKBR-3 cells. In contrast to the monovalent 800E6 scFv which bound erbB-2 at nearly equimolar concentrations as 800E6 Fab fragments, bispecific antibody preparations were 10 to 25-fold less efficient than unlabeled Fab fragments. A possible explanation for the observed reduction in binding is that a significant fraction of the scFv<sub>2</sub> preparation is not properly folded.

**Figure 3. Binding of 1B2/800E6 scFv<sub>2</sub> erbB2 on the human tumor line SKBR-3.** HPLC purified scFv<sub>2</sub> or crude scFv<sub>2</sub> were compared with unlabeled Fab fragments for their ability to inhibit binding of <sup>125</sup>I-labeled 800E6 Fab fragments.

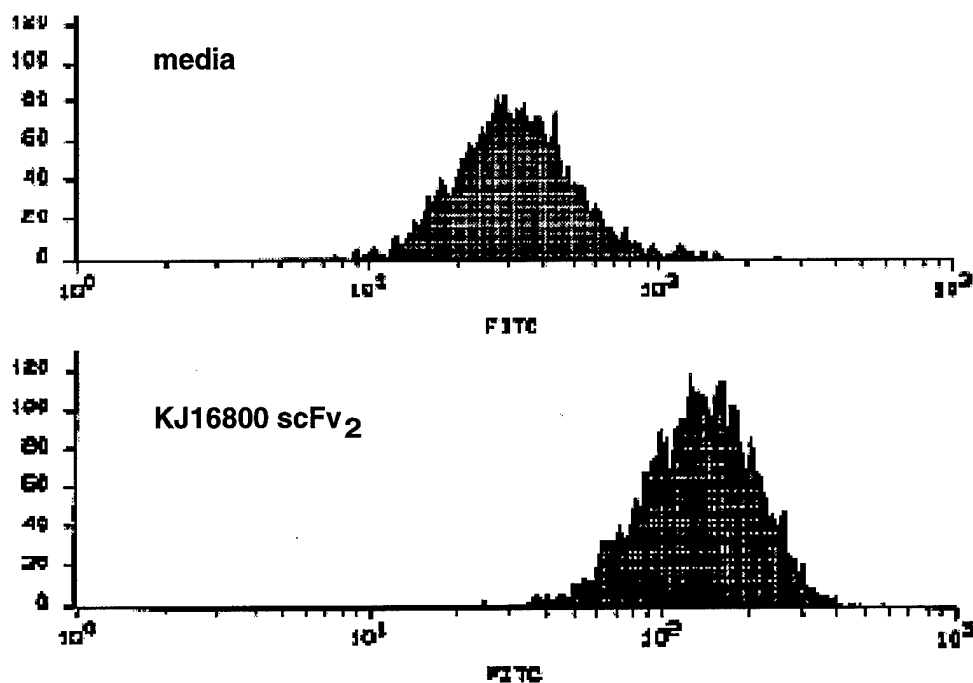


The KJ16/800E6 scFv<sub>2</sub> was also examined in an ELISA for binding to soluble T cell receptor absorbed to 96-well plates (Figure 4). This antibody (but not the 1B2 scFv<sub>2</sub>, data not shown) was found to bind specifically to the soluble TCR and to the cell surface of CTL 2C by flow cytometry (data not shown). At this time it is unclear why detectable binding of the 1B2 scFv<sub>2</sub> was not detected, although it is likely due to the less efficient folding of this scFv domain compared to the KJ16 scFv domain. In addition, the KJ16/800E6 scFv<sub>2</sub> bound to erbB-2+ tumor cell line, BT474 as judged by flow cytometry (Figure 5).

**Figure 4. Binding of KJ16/800E6 scFv<sub>2</sub> to soluble 2C scTCR.** ELISA results using soluble TCR adsorbed to 96 well plates, followed by various dilutions of the scFv<sub>2</sub>. Binding was detected with a monoclonal antibody to c-myc followed by HRP-labeled anti-mouse IgG.



**Figure 5. Binding of KJ16/800E6 scFv<sub>2</sub> to the tumor line BT-474.** Flow cytometry of the erbB-2<sup>+</sup> tumor BT474 incubated with either media or scFv<sub>2</sub>, followed by a mouse monoclonal antibody to c-myc and fluorescein-labeled anti-mouse IgG.





**Bispecific Antibody Activity in CTL-Mediated Killing Assays.** As the scFv<sub>2</sub> had erbB-2 binding activity, it was of interest to examine if the antibodies could redirect CTL 2C to lyse target cells. At this time we have only tested the 1B2 scFv<sub>2</sub> but will soon complete studies with KJ16 scFv<sub>2</sub>. This was of particular interest because no one has yet reported redirected killing with an anti-erbB-2 scFv<sub>2</sub> and it was possible that this property could be influenced by the nature of the inter-scFv linker. As expected, the L<sup>d</sup> alloreactive CTL 2C was unable to lyse human, erbB-2 positive breast carcinoma line BT-474 without the bispecific antibody. However, BT-474 were lysed when 1B2/800E6 scFv<sub>2</sub> was added to the CTL/target cell assay (Table 1). The observed lysis was dependent on the concentration of bispecific antibody (Table 1, Exp. 2), and could be inhibited with monospecific 1B2 or 800 intact Ab (Table 1, Exp. 1). The ability to mediate lysis requires covalent linkage of the two antibody binding domains as evidenced by the inability of a mixture of 1B2 and 800E6 monospecific antibodies to redirect lysis (Table 1, Exp.1).

**Table 1. Redirected lysis of erbB-2+ tumor cells by the 1B2/800E6 scFv<sub>2</sub>.**

Experiment 1	<u>antibody</u>	<u>μg/ml</u>	<u>Inhibitor</u>	<u>% Specific <sup>51</sup>Cr Release</u> <u>BT-474</u>
	-	-	-	0
	1B2 Ig +	25 + 25	-	4
	800E6 Ig			
	scFv <sub>2</sub>	13	-	22
	scFv <sub>2</sub>	13	25 μg/ml	0
			1B2 Ig	
	scFv <sub>2</sub>	13	25 μg/ml 800E6	0
			Ig	
	scFv <sub>2</sub>	13	-	0*
Experiment 2	<u>antibody</u>	<u>μg/ml</u>	<u>% Specific <sup>51</sup>Cr Release</u> <u>BT-474</u>	
	scFv <sub>2</sub>	5	20	
	scFv <sub>2</sub>	2.5	21	
	scFv <sub>2</sub>	1.3	18	
	scFv <sub>2</sub>	0.6	6	

\*In this assay, target cells were added to wells with media and scFv<sub>2</sub>, without effector cells. In all other wells 2C CTLs were added at an effector to target cell ratio of 10:1. All assays were performed in triplicate and standard error was less than ± 3%.

The observation that the 1B2 scFv<sub>2</sub> could redirect lysis was encouraging in light of the finding that both the anti-TCR and anti-erbB2 domains appear to be only partially folded (<5% by our estimates). Thus, the relatively high concentrations of scFv<sub>2</sub> required for activity (>1 ug/ml) might reasonably expect to be reduced if we can obtain fully folded forms of the scFv<sub>2</sub>. In addition, the KJ16 scFv<sub>2</sub> may be more effeciently folded based on the anti-TCR results described above. Our future efforts toward each of these aims are described below.

### **Specific Aim 3. Develop an *in vivo* model for targeting transplanted human breast cancer cells using immunodeficient, T cell receptor transgenic mice.**

The breeding, maintenance, and testing of immunodeficient, T cell receptor transgenic mice was described in last year's report. A continuous colony of TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice that express a monoclonal population of CD8<sup>+</sup> T cells with the TCR from CTL clone 2C has now been established. Peripheral lymphocytes (blood and spleen) and thymocytes from TCR<sup>+/+</sup>RAG<sup>-/-</sup> have been analyzed by flow cytometry in order to characterize their CD4, CD8 and TCR phenotypes. In addition, splenocytes have been cultured *in vitro* to show that they can be activated to produce a cytolytic population. Finally, two human tumor cell lines have now been successfully transplanted into the TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice. Each of these studies are described below.

#### **Phenotype of Peripheral Lymphocytes and Thymocytes from TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice.**

The affinity of the TCR for MHC class I or II is thought to direct the effector fate of the T cell toward a CD4<sup>+</sup> helper or CD8<sup>+</sup> cytotoxic T cell phenotype. Since the TCR/RAG<sup>-/-</sup> mice express only the 2C TCR, lymphocyte subsets were examined to determine if a monoclonal population of T cells would express only CD8 (like the 2C CTL clone). Spleen, thymus, and blood samples were analyzed for CD4, CD8, Thy1.2, and 2C TCR using multiple color flow cytometry (Figure 6, blood data not shown). Males and females ages 1-6 months were examined, with very similar results at all ages (data not shown). TCR/RAG<sup>-/-</sup> thymocytes were skewed toward a CD8<sup>+</sup>4<sup>-</sup> population. In contrast to C57Bl/6 normal mice in which >95% thymocytes are CD4<sup>+</sup>8<sup>+</sup>, TCR/RAG<sup>-/-</sup> thymocytes were on average 49% CD8<sup>+</sup>4<sup>-</sup>, 45% CD4<sup>+</sup>8<sup>+</sup>, and 5% CD4<sup>+</sup>8<sup>-</sup> (Figure 6).

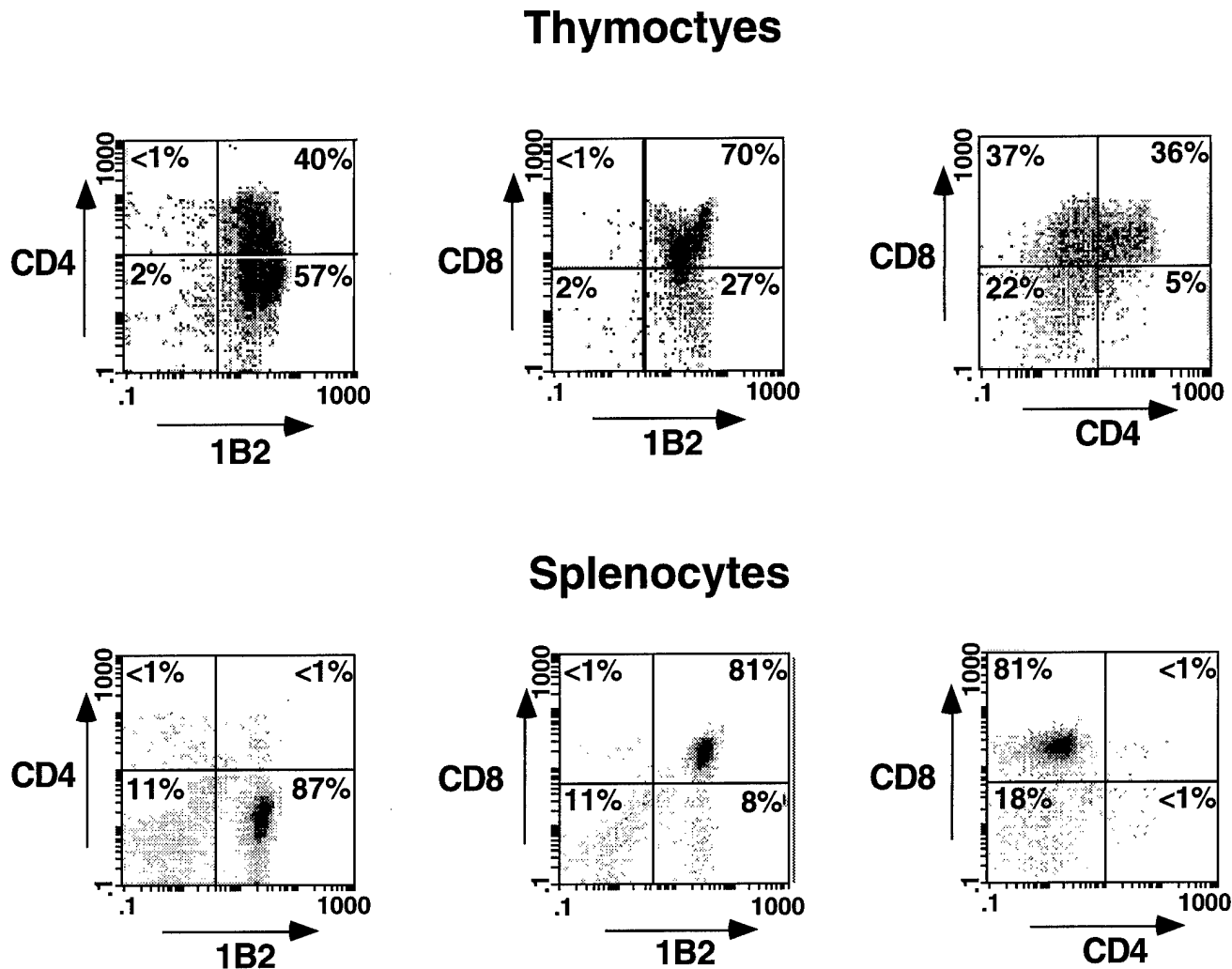
The skewing toward a CD8<sup>+</sup> phenotype was dramatic in the peripheral T cells of the TCR/RAG<sup>-/-</sup> mouse where >98% were CD8<sup>+</sup>4<sup>-</sup> (Figure 6). This is in contrast to C57Bl/6 normal mice which had 60% CD4<sup>+</sup>8<sup>-</sup> and 40% CD8<sup>+</sup>4<sup>-</sup>. Although splenocytes from 2C TCR transgenic mice were also skewed toward CD8<sup>+</sup>4<sup>-</sup> (~70%), there were a significant number of CD4<sup>+</sup>CD8<sup>-</sup> T cells (~30%) in the periphery (Gruber and Kranz, unpublished results; ref 19). In contrast, the TCR/RAG<sup>-/-</sup> mice expressed very low to undetectable levels of CD4<sup>+</sup>8<sup>-</sup> T cells (0-5%), with nearly all T cells being 1B2<sup>+</sup>CD8<sup>+</sup>4<sup>-</sup> (93-99%) (Figure 6).

#### ***In Vitro* Activation of Peripheral Lymphocytes from TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice.**

Most peripheral T cells exist in a resting state in which they can not secrete cytokines or lyse target cells. Even though the TCR/RAG<sup>-/-</sup> mice displayed a monoclonal population of CD8<sup>+</sup> T cells, as might have been anticipated, these CTLs must have the ability to be activated, proliferate, and lyse target cells in order to be useful in immunotherapy.

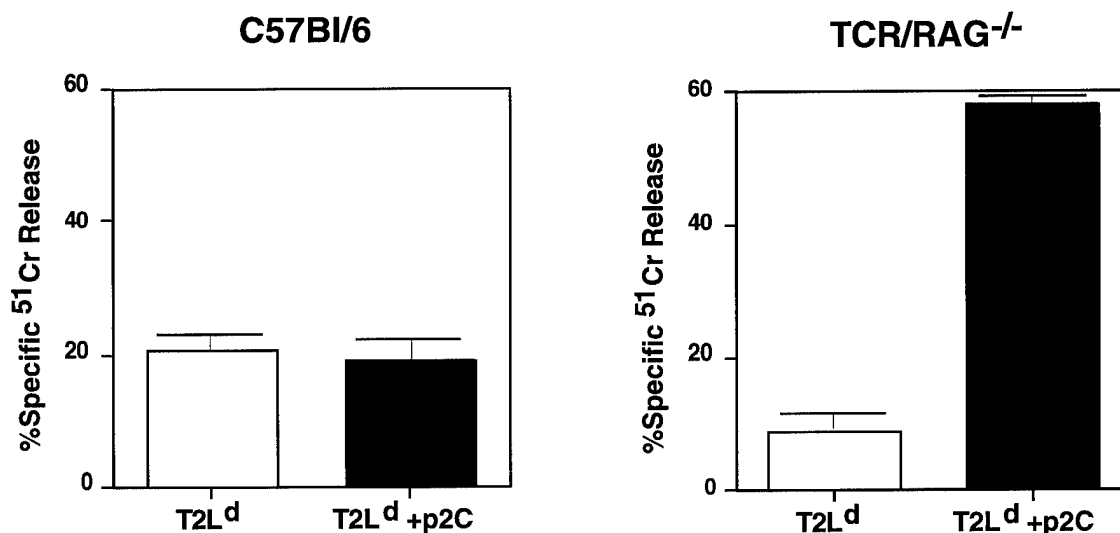
CTL activation was examined by incubation of the TCR/RAG<sup>-/-</sup> splenocytes with the superantigen, staphylococcal enterotoxin B (SEB). SEB has been used in many studies as an *in vitro* and *in vivo* T cell activating agent, and was therefore of interest for use with the TCR/RAG<sup>-/-</sup> mice. SEB has been shown to bind the  $\beta$  chain of TCRs that contain a V $\beta$ 8 TCR gene product (23). SEB binds to the TCR and to a MHC class II molecule, which allows activation of the CTL regardless of peptide/MHC class I specificity (24). Although ~20% of T cells in a normal mouse will express V $\beta$ 8 TCRs, all of the CTLs from the TCR/RAG<sup>-/-</sup> mouse express V $\beta$ 8.2 and thus should be responsive to SEB activation, if sufficient IL-2 is present as a growth factor.

**Figure 6. Flow cytometric analysis of thymic and splenic lymphocyte subsets from a TCR/RAG<sup>-/-</sup> mouse.** 1B2 is a monoclonal antibody that is specific only for the TCR derived from CTL 2C. 1B2 was directly labeled with fluorescein isothiocyanate; anti-CD4 was labeled with phycoerythrin; anti-CD8 was labeled with cychrome.



To test for the specific activation of 2C TCR<sup>+</sup> CTLs from the TCR/RAG<sup>-/-</sup> spleen, a chromium release assay was performed with the activated splenocytes. The target cell was a human cell line transfected with MHC class I haplotype, L<sup>d</sup>, called T2L<sup>d</sup>. This cell line expresses L<sup>d</sup> from the transfected gene, but does not possess the mouse peptide, p2C (25). Incubation of T2L<sup>d</sup> with the peptide, p2C will "load" the L<sup>d</sup> MHC, providing the alloantigen which is recognized by the 2C TCR. T2L<sup>d</sup> without the peptide serves as a control for 2C TCR specific lysis as it shows the amount of lysis from cells which lyse independent of p2C/L<sup>d</sup>. SEB activated p2C/L<sup>d</sup>-specific CTLs that were able to lyse T2L<sup>d</sup> only after addition of the peptide, p2C (Figure 7). As mentioned, ~20% of C57Bl/6 T cells would be expected to respond to SEB activation. However, very few of these CTLs express a TCR that is specific for the p2C/L<sup>d</sup> complex. A moderate amount of lysis was observed for T2L<sup>d</sup> with and without the peptide, p2C. This non-specific lysis may be due to the induction of cytolytic cells that have no MHC/peptide restriction or that recognize L<sup>d</sup> without the specific peptide, p2C.

**Figure 7. SEB mediated activation of CTLs from TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice.** Spleen cells from a normal C57Bl/6 mouse and a TCR/RAG mouse were stimulated for 3 days *in vitro* and assayed for cytotoxicity using the target cell for the 2C CTL clone, T2L<sup>d</sup> and the peptide p2C.



In conclusion, the TCR/RAG<sup>-/-</sup> mice express a monoclonal population of CD8<sup>+</sup> T cells that are capable of being activated *in vitro*. These activated T cells lyse target cells that express only the specific alloantigen (p2C/L<sup>d</sup>).

#### **Xenografts of Human Tumors in TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice.**

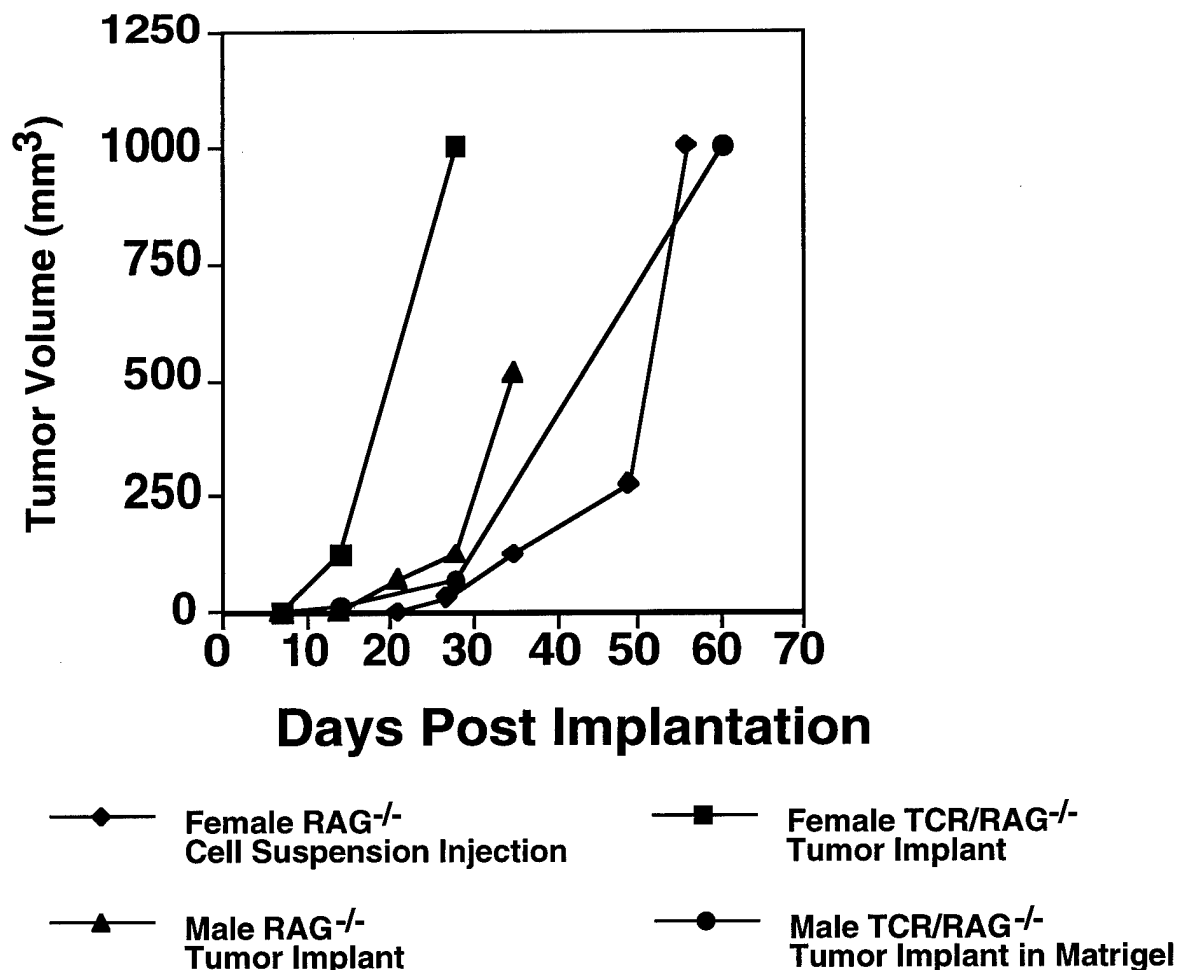
Human erbB-2<sup>+</sup> tumors were introduced into TCR/RAG<sup>-/-</sup> mice to evaluate if the transgenic animals could serve as a model for immunotherapy of erbB-2<sup>+</sup> cancers. SKOV-3, an erbB-2<sup>+</sup> ovarian carcinoma, was chosen initially because it was susceptible to CTL-mediated lysis and because several labs have used SKOV-3 in immunodeficient mice as a model for immunotherapy (13, 16). Because xenotransplantation in RAG<sup>-/-</sup> mice had not been reported, the SKOV-3 tumor was initially transplanted into RAG<sup>-/-</sup> mice. Of eleven RAG<sup>-/-</sup> mice injected with 2-10x10<sup>6</sup> cells from tissue culture, only two had established grafts (data not shown). The reason for the inability of the tumor to take in nine of the mice was not explored. However, seven TCR/RAG<sup>-/-</sup> received tumor explants from the two *in vivo* tumors from RAG<sup>-/-</sup> mice and all established grafts (Figure 8 and data not shown). Tumors were palpable 10-20 days post injection and grew to a volume of 1000mm<sup>3</sup> (1cm diameter) in approximately three weeks, at which point animals were sacrificed.

The BT-474 human breast adenocarcinoma also expresses a high level of erbB-2, was lysed by CTL 2C (Gruber and Kranz, unpublished), and has been used in SCID mice for animal models of breast cancer (26). Furthermore, BT-474 expresses estrogen receptors and has been used as a model for estrogen therapies. Thus, combination immunotherapy and hormonal therapies could be envisioned with this tumor line. To establish BT-474 grafts, RAG<sup>-/-</sup> and TCR/RAG<sup>-/-</sup> mice were implanted with estradiol pellets because of the estrogen-dependent growth of BT-474. Matrigel basement membrane has been used to enhance tumor take, and was shown to be beneficial when injected with BT-474 (27). Cells were injected at a ratio of 1:1 with Matrigel basement

membrane matrix. Of the two males and two females injected, one BT-474 transplantation was successful in a TCR/RAG<sup>-/-</sup> female.

Together, these results demonstrate that the TCR/RAG<sup>-/-</sup> mice are capable of accepting erbB-2+ human tumor xenografts. Thus, this animal model should be useful in testing bispecific antibody agents, in the continuation of studies proposed for this project.

**Figure 7. SKOV-3 and BT474 xenograft growth in TCR+/+RAG<sup>-/-</sup> mice.**



### CONCLUSIONS

Tasks shown below with an \* have now been completed and significant progress was made on tasks indicated with an \*\*:

**\*Task 1,** Cloning, expression, and testing of erbB-2 single-chain antibody, months 1-12.

**\*Task 2,** Screening of tumor cell lines for susceptibility to CTL-mediated lysis using the anti-fluorescein bispecific antibody, months 1-12.

**\*Task 3,** Breeding of transgenic TCR/RAG-2<sup>-</sup> mice and testing of peripheral blood T cells for reactivity with 1B2 antibodies, months 1-20. It is anticipated that approximately 175 mice will be produced by the end of this period.

**\*Task 4**, Cloning, expression, and *in vitro* testing of bispecific single-chain scFv<sub>2</sub> antibody (1B2/erbB-2), months 13-24.

**\*Task 5**, Screening of tumor cell lines for increased susceptibility to CTL-mediated lysis when tumor cells are treated with: anti-erbB-2 antibodies, IFN- $\gamma$ , TNF- $\alpha$ , estrogen, tamoxifen, months 13-30.

**\*\*Task 6**, Transplantation of various erbB-2<sup>+</sup> tumor cell lines into TCR/RAG-2<sup>-</sup> mice and evaluation of tumor incidence, months 16-36. It is anticipated that approximately 15 mice per month will be used.

**\*\*Task 7**, Purification and *in vitro* testing of bispecific Fab<sub>2</sub> antibody (1B2/erbB-2), months 25-36.

**Task 8**, *In vivo* testing of bispecific antibodies in TCR/RAG-2<sup>-</sup> mice that have received human tumor transplants, months 30-48. It is anticipated that approximately 15 mice per month will be used.

**Publications of the Principle Investigator During the Past Year (\*Directly related to this project):**

Schlueter, C. J., B. A. Schodin, and D. M. Kranz (1996) Specificity and Binding Properties of a Single-Chain T Cell Receptor. *J. Mol. Biol.* 256:859-869.

Brodnicki, T., P.O. Holman, and D. M. Kranz (1996) Reactivity and Epitope Mapping of Single-Chain T Cell Receptors with Monoclonal Antibodies. *Mol. Immunol.* 33:253-263.

Manning, T.C., B.A. Schodin, and D.M. Kranz (1996) A Strategy for the Synthesis and Screening of Thiol-Modified Peptide Variants Recognized by T Cells. *J. Immunol. Meth.* 192:125-132.

Schodin, B.A., T. J. Tsomides, and D. M. Kranz (1996) Correlation Between the Number of T Cell Receptors Required for T Cell Activation and TCR-Ligand Affinity. *Immunity* 5:

Patrick, T. A., D. M. Kranz, T. A. Van Dyke, and E. J. Roy (1996) Folate Receptors as Potential Therapeutic Targets in Choroid Plexus Tumors of SV40 Transgenic Mice. *J. Neuro. Oncol.* In Press.

Schodin, B. A., C. J. Schlueter, and D. M. Kranz (1996) Binding Properties and Solubility of Single-Chain T Cell Receptors Expressed in *E. coli*. *Mol. Immunol.* In Press.

Schlueter, C. J., T. C. Manning, B. A. Schodin, and D. M. Kranz (1996) A Residue in the Center of Peptide QL9 Affects Binding to Both L<sup>d</sup> and the T Cell Receptor. *J. Immunol.* In Press

\*Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1996) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. Submitted.

\*Gruber, M. M., P. G. Natali, and D. M. Kranz (1996) Lysis of Breast Cancer Cells Mediated by a Bispecific Single-Chain Anti-erbB-2 Antibody. Submitted.

\*Gruber, M. M. and D. M. Kranz (1996) A Combination of  $\gamma$ -IFN and TNF- $\alpha$  Can Increase the Susceptibility of erbB-2 Positive Tumor Cells to CTL-Mediated Lysis. Submitted.

## Future Work:

During the next year, we intend to complete the tasks indicated above by an \*\* and to begin three new tasks that we believe are important for the success of the project. These tasks will address the important questions of whether a more effective scFv<sub>2</sub> can be obtained (Tasks 9 and 10) and whether it is possible to activate CTLs in the TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice *in vivo* (Task 11).

### Task 9. Characterization of the KJ16/800E6 (anti-V $\beta$ 8/anti-erbB2) bispecific scFv<sub>2</sub>.

As described above, preliminary results suggest this scFv<sub>2</sub> may have higher fraction of properly folded antibodies after refolding from *E. coli* inclusion bodies, than the 1B2/800E6 scFv<sub>2</sub>. This scFv<sub>2</sub> will thus be further analyzed for its ability to retarget erbB2<sup>+</sup> tumor cells *in vitro*.

### Task 10. Expression of scFv<sub>2</sub> (1B2/800E6 and KJ16/800E6) in a yeast secretion system.

In collaboration with Professor Dane Wittrup at the University of Illinois, we have begun to clone the scFv<sub>2</sub> in an expression vector that has been shown to allow a high yield of active scFv to be secreted. The yeast expression system is predicted to allow only a properly folded protein to be secreted and thus we hope to be able to obtain a much higher fraction of active scFv<sub>2</sub> than in the *E. coli* system.

### Task 11. Comparison of *in vivo* activation strategies in TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice.

Our progress in xenotransplantation of human tumors in TCR<sup>+/+</sup>RAG<sup>-/-</sup> mice now dictates that we must decide on a method for activating T cells *in vivo* prior to bispecific antibody treatments. Three different agents will be evaluated in this effort: the anti-CD3 antibody 2C11, the superantigen Staphylococcal enterotoxin B, and a peptide (SIYRYYYGL) that has recently been shown to activate the CTL clone 2C and thus, we presume, the TCR transgenic T cells (28).

## REFERENCES

1. Schechter, A. L., M.-C. Hung, L. Vaidyanathan, R. A. Weinberg, T. L. Yang-Feng, A. Ullrich, L. Coussens. 1985. The *neu* Gene: An *erbB*-Homologous Gene Distinct from and Unlinked to the Gene Encoding the EGF Receptor. *Science*. 229:976-978.
2. Bargmann, C. I., M.-C. Hung, and R. A. Weinberg. 1986. The *neu* Oncogene Encodes an Epidermal Growth Factor Receptor-Related Protein. *Nature*. 319:226-230.
3. King, C. R., M. H. Kraus, S. A. Aaronson. 1985. Amplification of a Novel *v-erbB*-Related Gene in a Human Mammary Carcinoma. *Science*. 229:974-976.
4. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, W. L. McGuire. 1987. Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/*neu* Oncogene. *Science*. 235:177-182.
5. McCann, A. H., P. A. Dervan, M. O'Regan, M. B. Codd, W. J. Gullick, B. M. J. Tobin, and D. N. Carney. 1991. Prognostic Significance of *c-erbB-2* and Estrogen Receptor Status in Human Breast Cancer. *Cancer Res*. 51:3296-3303.

6. **Pietras, R. J., J. Arboleda, D. M. Reese, N. Wongvipat, M. D. Pegram, L. Ramos, C. M. Gorman, M. G. Parker, M. X. Sliwkowski, and D. J. Slamon.** 1995. HER-2 Tyrosine Kinase Pathway Targets Estrogen Receptor and Promotes Hormone-Independent Growth in Human Breast Cancer Cells. *Oncogene* 10:2435-2446.
7. **Hudziak, R. M., G. D. Lewis, M. R. Shalaby, T. E. Eessalu, B. B. Aggarwal, A. Ullrich, and H. M. Shepard.** 1988. Amplified Expression of the HER2/ERBB2 Oncogene Induces Resistance to Tumor Necrosis Factor  $\alpha$  in NIH 3T3 Cells. *Proc. Natl. Acad. Sci. USA.* 85:5102-5106.
8. **Lichtenstein, A., J. Berenson, J. F. Gera, K. Waldburger, O. Martinez-Maza, and J. S. Berek.** 1990. Resistance of Human Ovarian Cancer Cells to Tumor Necrosis Factor and Lymphokine-activated Killer Cells: Correlation with Expression of HER2/*neu* Oncogenes. *Cancer Res.* 50:7364-7370.
9. **McKenzie, S. J., P. J. Marks, T. Lam, J. Morgan, D. L. Panicali, K. L. Trimpe, and W. P. Carney.** 1989. Generation and Characterization of Monoclonal Antibodies Specific for the Human *neu* Oncogene Product, p185. *Oncogene.* 4:543-548.
10. **Hancock, M. C., B. C. Langton, T. Chan, P. Toy, J. J. Monahan, R. P. Mischak, and L. K. Shawver.** 1991. A Monoclonal Antibody Against the *c-erbB-2* Protein Enhances the Cytotoxicity of cis-Diamminedichloroplatinum against Human Breast and Ovarian Tumor Cell Lines. *Cancer Res.* 51:4575-4580.
11. **Digiesi, G., P. Giacomini, R. Fraioli, M. Mariani, M. R. Nicotra, O. Segatto, and P. G. Natali.** 1992. Production and Characterization of Murine mAbs to the Extracellular Domain of Human Neu Oncogene Product GP185<sup>HER2</sup>. *Hybridoma.* 11:519-527.
12. **Lewis, G. D., I. Figari, B. Fendly, W. L. Wong, P. Carter, C. Gorman, H. M. Shepard.** 1993. Differential Responses of Human Tumor Cell Lines to Anti-p185<sup>HER2</sup> Monoclonal Antibodies. *Cancer Immunol. Immunother.* 37:255-263.
13. **Adams, G. P., J. E. McCartney, M.-S. Tai, H. Oppermann, J. S. Huston, W. F. Stafford III, M. A. Bookman, I. Fand, L. L. Houston, and L. M. Weiner.** 1993. Highly Specific *in Vivo* Tumor Targeting by Monovalent and Divalent Forms of 741F8 Anti-*c-erbB-2* Single-Chain Fv. *Cancer Res.* 53:4026-4034.
14. **De Santes, K., D. Slamon, S. K. Anderson, M. Shepard, B. Fendly, D. Maneval, and O. Press.** 1992. Radiolabeled Antibody Targeting of the HER-2/*neu* Oncoprotein. *Cancer Res.* 52:1916-1923.
15. **Shalaby, M. R., H. M. Shepard, L. Presta, M. L. Rodrigues, P. C. L. Beverley, M. Feldmann, and P. Carter.** 1992. Development of Humanized Bispecific Antibodies Reactive with Cytotoxic Lymphocytes and Tumor Cells Overexpressing the *HER2* Protooncogene. *J. Exp. Med.* 175:217-225.
16. **Weiner, L. M., M. Holmes, G. P. Adams, F. LaCreta, P. Watts, and I. Garcia de Palazzo.** 1993. A Human Tumor Xenograft Model of Therapy with a Bispecific Monoclonal Antibody Targeting *c-erbB-2* and CD16. *Cancer Res.* 53:94-100.
17. **Gruber, M., B. Schodin, E. Wilson, and D. M. Kranz.** 1994. Efficient Tumor Cell Lysis Mediated by a Bispecific Single-Chain Antibody Expressed in *E. coli*. *J. Immunol.* 152, 5368-5374.



18. **Schodin, B. A. and D. M. Kranz.** 1993. Binding Affinity and Inhibitory Properties of a Single-Chain Anti-T Cell Receptor Antibody. *J. Biol. Chem.* 268:25722-25727.
19. **Sha, W. C., C. A. Nelson, R. D. Newbury, D. M. Kranz, J. H. Russell, and D. Y. Loh.** 1988. Selective Expression of an Antigen Receptor on CD8-bearing T Lymphocytes in Transgenic Mice. *Nature* 335, 271-274.
20. **Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Russell, and D. Y. Loh.** 1988. Positive and Negative Selection of an Antigen Receptor on T Cells in Transgenic Mice. *Nature.* 336:73-76.
21. **Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou.** 1992. RAG-1 Deficient Mice Have No Mature B and T Lymphocytes. *Cell* 68:869-878.
22. **Kranz, D. M., S. Tonegawa, and H. N. Eisen.** 1984. Attachment of an Anti-Receptor Antibody to Non-Target Cells Renders Them Susceptible to Lysis by a Clone of Cytotoxic T Lymphocytes, *Proc. Natl. Acad. Sci. USA* 81, 7922-7926.
23. **Haskins, K., Hannum, C., White, J., Roehm, N., Kubo, R., Kappler, J., and Marrack, P.** 1984. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. *J. Exp. Med.* 160: 452-471.
24. **Herman A, Kappler JW, Marrack P, Pullen AM.** 1991. Superantigens: Mechanism of T-cell stimulation and role in immune responses. *Annu. Rev. Immunol.* 9: 745-772.
25. **Udaka, K., Tsomides, T.J. & Eisen, H.N.** (1992). A naturally occurring peptide recognized by alloreactive CD8+ cytotoxic T lymphocytes in association with a class I MHC protein. *Cell* **69**, 989-998.
26. **Park, J. W. K. Hong, P. Carter.** 1995. Development of anti-p185 HER2 immunoliposomes for cancer therapy. *Proc. Natl. Acad. Sci. USA* 92: 1327-1332.
27. **Van Slooten, H. J., B. A. Bonsing, A. J. Hiller.** 1995. Outgrowth of BT474 human breast cancer cells in immunodeficient mice: a new *in vivo* model for hormone-dependent breast cancer. *Brit. J. Can.* 72: 22.
28. **Udaka, K., K.H. Wiesmuller, S. Kienle, G. Jung, and P. Walden.** 1996. Self-MHC-restricted peptides recognized by an alloreactive T lymphocyte clone. *J. Immunol.* 157: 670-678.

## **Appendix**

**Three manuscripts that involve this project are provided in the Appendix:**

Cho, B.K., T.A. Patrick, E.J. Roy, and D. M. Kranz (1996) Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells by Single-Chain Fv/Folate Conjugates. Submitted.

Gruber, M. M., P. G. Natali, and D. M. Kranz (1996) Lysis of Breast Cancer Cells Mediated by a Bispecific Single-Chain Anti-erbB-2 Antibody. Submitted.

Gruber, M. M. and D. M. Kranz (1996) A Combination of  $\gamma$ -IFN and TNF- $\alpha$  Can Increase the Susceptibility of erbB-2 Positive Tumor Cells to CTL-Mediated Lysis. Submitted.

**Efficient CTL Lysis of Folate-Receptor-Positive Tumor Cells Mediated by  
Single-Chain Fv/Folate Conjugates<sup>1</sup>**

Bryan K. Cho,<sup>\*</sup> Edward J. Roy,<sup>‡†</sup> Todd A. Patrick,<sup>†</sup> and David M. Kranz<sup>\*2</sup>

<sup>\*</sup>University of Illinois Department of Biochemistry, <sup>‡</sup>Department of Psychology,

<sup>†</sup>Neuroscience Program, Urbana, IL 61801

**Running Title: Tumor Targeting with Antibody/Folate Conjugates**

## Abstract

Bispecific antibodies that bind to a tumor antigen and the T cell receptor (TCR) redirect cytotoxic T lymphocytes (CTL) to lyse tumor cells which have escaped normal immune recognition mechanisms. One well-characterized tumor antigen, the folate receptor (FR), is expressed on most ovarian carcinomas and some types of brain cancer. Recently, we showed that conjugates of folate and anti-TCR antibodies are extremely potent bispecific agents that target tumor cells expressing the high affinity folate receptor, but not normal cells expressing only the reduced folate carrier protein. In this report, we show that the size of these conjugates can be reduced to the smallest bispecific agent yet described (30-kDa) by attaching folate to a single-chain antibody, scFv, of the anti-TCR antibody KJ16. The scFv/folate conjugates are as effective as IgG/folate conjugates in mediating lysis of FR<sup>+</sup> tumor cells by CTL. The optimal folate density was in the range of 5 to 15 folate molecules per scFv or IgG molecule, which yielded EC<sub>50</sub> values of approximately 40 pM (1.2 ng/ml for scFv). Finally, the scFv/folate conjugates could efficiently target tumor cells even in the presence of free folic acid at concentrations that are normally found in serum. Compared to conventional bispecific antibodies, the small size of scFv/folate conjugates may prove advantageous in the ability to penetrate tumors and in reduced immunogenicity.

## Introduction

It has been known for over fifty years that the immune system is capable of attacking and eliminating very large tumor burdens but sometimes fails to do so (1). Although the basis of this "escape" is incompletely understood (2), one mechanism involves the failure of tumor cells to express antigens in a context that is essential for recognition by the immune system (reviewed by Pardoll (3)). Another mechanism might be the loss of co-stimulatory ligands and adhesion molecules that aid in the recognition and activation of T cells (4).

One potential way to direct T cells or other immune effector cells against tumor cells is with bispecific antibodies (reviewed by Fanger (5)). Bispecific antibodies can be constructed to recognize two separate antigens, one on the tumor surface and the other on the surface of a cytotoxic T cell (e.g. TCR<sup>3</sup>). Many tumor cells have potential target antigens that are tumor-specific or quantitatively more abundant on tumor cells than normal cells (tumor-associated). By bringing together the tumor cell and an activated T cell, bispecific antibodies can redirect the cytotoxicity of T cells against tumors. Previous work has demonstrated the effectiveness of bispecific antibodies against tumors *in vitro* and *in vivo* and some clinical trials have been initiated (e.g. see Refs. (6-11)). It has generally been agreed that optimizing the properties of bispecific antibodies should improve their clinical effectiveness.

Among the tumor antigens targeted with bispecific antibodies has been the high affinity folate receptor. This receptor was originally identified as a tumor-associated antigen using monoclonal antibodies that reacted with ovarian tumor cell lines (12). High affinity folate receptors (FR) are now known to be expressed at elevated levels on many human tumors, including ovarian carcinomas (e.g. one study showed that 98% of ovarian tumors express the FR, ref (13)), choroid plexus carcinomas and ependymomas (14, 15). These cancers affect a significant segment of the population: ovarian cancer is the fourth

leading cause of cancer death among women (16) and at least 30% of early childhood tumors are diagnosed as ependymoma or choroid plexus tumors (17, 18).

The high affinity FRs ( $K_D \sim 1$  nM) differ from the lower affinity reduced folate carriers ( $K_D \sim 100$   $\mu$ M) that appear to be largely responsible for normal folate uptake as well as the transport of folate-based dihydrofolate reductase inhibitors such as methotrexate (MTX, (19-21)). The presence of FR on human tumor cells has led to the use of FR as a target for various forms of therapy with monoclonal antibodies such as MOv18 and MOv19 (12). Targeting approaches with monoclonal anti-FR antibodies have included:  $^{131}\text{I}$ -labeled antibodies (22), engineering of constant regions to optimize antibody-dependent cellular cytotoxicity (23), and bispecific antibodies that target immune effector cells to the FR<sup>+</sup> tumor (24-26). The latter studies have used anti-FR antibodies linked to either anti-Fc receptor antibodies or to anti-CD3 antibodies for recruitment of monocyte/natural killer cells or cytotoxic T cells, respectively. Clinical trials with the radiolabeled antibodies and the anti-FR/anti-CD3 bispecific antibodies have recently been initiated (9, 11, 22). Delivery of toxins and antisense nucleotides to the interior of malignant cells using the endocytotic properties of the FR have also been undertaken (27, 28).

Monoclonal antibodies to the mouse FR have not been produced and thus antibody targeting of the FR on mouse tumor cells as a model for human disease has not been possible. Nevertheless, two mouse homologs of the human FR isoforms have been identified and these receptors also bind folate with high affinity ( $K_D \sim 1$  nM) (29, 30). Several mouse leukemia lines have been selected for high FR expression by growth in media with low concentrations of folate. Two forms ( $\alpha$  and  $\beta$ ) of the mouse FR have been identified as 38-kDa lipid-linked membrane glycoproteins (31). As in humans, FR also appear to be expressed at high levels on some mouse tumors. For example, we have recently found that mouse choroid plexus tumors that arise in SV40 transgenic mice (32) express high levels of FR (33).

The high affinity of folate for FR suggested to us that attachment of folate directly to an anti-TCR antibody might efficiently target FR<sup>+</sup> tumor cells for lysis by T cells. We recently reported that such conjugates have very potent targeting activity without adversely affecting normal cells that express only the reduced folate carrier protein (34). However, it is reasonable to predict that the most effective agents for targeting solid tumors will have reduced sizes that allow greater tumor penetration. For instance, comparative bio-distribution studies with <sup>125</sup>I-labeled IgG, F(ab)<sub>2</sub>, Fab fragments and scFv in human colon carcinoma xenografts in athymic mice demonstrated that scFv penetrated tumor faster, deeper, and more uniformly than other forms of the antibody (35).

In this report, a 29-kDa scFv of the anti-V $\beta$ 8 antibody KJ16 (36) was conjugated with folate, and its targeting potential was evaluated *in vitro*. Cytotoxicity assays with these preparations showed that lysis of mouse FR<sup>+</sup> tumor cells was highly specific and correlated directly with FR density ( $r = 0.93$ ). Comparison between folate labeled-IgG and scFv demonstrated that both conjugates have nearly identical targeting efficiencies ( $EC_{50} = 40$  pM) and lysis with scFv/folate could be detected at concentrations as low as 1 pM. Direct competition experiments with free folate demonstrated that the scFv/folate conjugate could effectively target FR<sup>+</sup> tumor cells even at folate concentrations above normal serum levels. The reduced size of the scFv/folate compared to other bispecific reagents as well as its high potency suggests that it has potential for *in vivo* therapy. In addition, the conjugate may serve as a model for the development of future novel bispecific agents that contain small ligands specific for tumor cell surface antigens.

## Materials and Methods

**Cell Lines and Antibodies.** The following DBA/2-derived tumor cell lines were maintained in RPMI 1640 containing 5 mM HEPES, 10% fetal calf serum, 1.3 mM L-glutamine, 100 units of penicillin per ml, 100  $\mu$ g per ml streptomycin, and 50  $\mu$ M 2-mercaptoethanol: Mel, murine erythroleukemia cell (37); La, a subline of Mel selected on

low folate (31); L1210, a murine leukemia cell line (38); and F2-MTX<sup>r</sup>A, a MTX resistant subline of L1210 selected for increased expression of FR- $\beta$  by growth on low folic acid (39). La expresses primarily the  $\alpha$  isoform of folate receptor (FR), F2-MTX<sup>r</sup>A expresses only the FR- $\beta$  isoform, and L1210 expresses both  $\alpha$  and  $\beta$  isoforms. CTL clone 2C, a V $\beta$ 8<sup>+</sup> alloreactive cell line specific for L<sup>d</sup> (40), was maintained in the same RPMI media supplemented with 10% (vol/vol) supernatant from concanavalin A-stimulated rat spleen cells, 5%  $\alpha$ -methyl mannoside, and mitomycin-C treated BALB/c mouse spleen cells as stimulators. KJ16 is a rat IgG antibody specific for the V $\beta$ 8.1-2 domains of the TCR (41) and was provided by Drs. Kappler and Marrack. KJ16 monoclonal antibody was prepared from tissue culture supernatant generated in a Vita-Fiber mini flow path bioreactor (Amicon) and concentrated by precipitating twice in 50% ammonium sulfate. KJ16 Fab fragments, FITC-labeled Fab fragments, and KJ16 scFv were generated and purified as described previously (36). Briefly, scFv was refolded from inclusion bodies and monomeric scFv was purified by G-200 HPLC purification. Monoclonal antibody 30.5.7 is specific for the major histocompatibility complex (MHC) class I product L<sup>d</sup> (42) and was prepared as ascites fluid and used without further purification in some cytotoxicity assays.

**Preparation of Folate/Antibody Conjugates.** Folate was coupled through its carboxyl groups to antibody amine groups using a carbodiimide procedure described previously (34, 43). Unless indicated, a 5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, Pierce) was added to folate (Sigma) dissolved in dimethyl sulfoxide. After 30 minutes at room temperature in the dark, a 20- to 700- fold molar excess of the EDC-activated folate was added to 0.1 - 0.5 mg of antibody in 0.1 M MOPS, pH 7.5. After 1 hour at room temperature, the sample was either applied to a Sephadex G-25 column pre-equilibrated in 0.1 M MOPS or immediately dialyzed into phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.3). If passed over a G-25 column, the excluded-peak fractions were pooled and dialyzed against PBS. After dialysis, protein concentrations were determined using the



bicinchoninic assay (Pierce Chemical Co.) using a protein A purified intact mouse antibody as the standard. Antibody conjugates were also analyzed spectrophotometrically at 363 nm and the density of the folate per antibody was calculated by dividing the molar concentration of folate on the conjugate ( $A_{363}/\epsilon_M$ ;  $\epsilon_M = 6197 \text{ M}^{-1}$ ) by the antibody concentration. Folate densities obtained ranged from ~1 folate/antibody to ~120 folates/antibody. Conjugates were stored at 4°C in the dark.

**Mass Spectrometry.** Mass spectra were obtained on a ToFSpec using electrospray ionization. Samples were dialyzed against 1 mM potassium phosphate buffer, pH 8.0 and concentrated to 10 to 25 pmol/ml. Analysis was performed by the Mass Spectrometry Laboratory, School of Chemical Science, University of Illinois.

**Folate Binding Assays.** Binding assays were conducted by using  $^{125}\text{I}$ -labeled folate (NEN; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq). Cells were washed with PBS containing 0.1% bovine serum albumin, pH 7.3 (PBS-BSA), to remove excess free folate present in the cell culture medium. Cells, labeled folate, and competitors were incubated in triplicate in 50  $\mu\text{l}$  of PBS-BSA for 1 hour at 37°C. Incubation at 37°C has previously been shown to produce levels of binding similar to that obtained with acid pre-treatment (34). Samples were loaded into tubes containing 300  $\mu\text{l}$  oil [80% (vol/vol) dibutyl phthalate/20% (vol/vol) olive oil] and bound and free ligand were separated by a 3 second centrifugation at 12,000  $\times g$ . Tubes were frozen and cut to allow the radioactivity in the cell pellet and supernatants to be quantitated separately.

**T Cell Receptor Binding Assays.** The relative affinity of the scFv/folate conjugates for the TCR was determined by a competition assay with 5-aminofluorescein isothiocyanate (FITC) labeled KJ16 Fab fragments as previously described (36). In brief, various concentrations of antibody were added to triplicate sets of  $6 \times 10^5$  2C cells in the presence of a constant amount of FITC-labeled Fab fragments. After a 30 minute incubation on ice, the entire mixture (antibody + FITC-labeled Fab fragments + 2C cells) was passed through a flow cytometer without washing. Inhibition by various KJ16 preparations was measured

by quantitating the decrease in bound fluorescence by flow cytometry (performed with a Coulter Electronics EPICS 752 at the University of Illinois Biotechnology Center). The concentrations of unlabeled antibody giving 50% inhibition ( $IC_{50}$ ) were determined relative to the maximum fluorescence (in the absence of inhibitor) and the background fluorescence (in the presence of a large excess of intact antibody).

**Cytotoxicity Assays.** Tumor cells were labeled with 50-100  $\mu$ l of  $^{51}Cr$  (2.5 mCi/ml) for 60 minutes at 37°C, washed twice with folate-free RPMI 1640 medium containing 5% (vol/vol) fetal calf serum (folate-free media), and used in 96-well plate cytotoxicity assays at  $10^4$  cells per well. Because each of these cell lines also expressed the alloantigen  $L^d$  which is recognized by CTL 2C, assays were performed in the presence of anti- $L^d$  antibody to minimize non-FR-mediated lysis. Ascites of anti- $L^d$  antibody 30.5.7 was diluted 1:100 into folate-free media containing effector cells (2C). Effector cells were added to target cells at an effector-to-target cell ratio of 5:1. Antibodies and folate/antibody conjugates were diluted in folate-free media and added to triplicate wells at various concentrations. Plates were incubated at 37°C for 4 hours in 5%  $CO_2$ , and supernatants were removed for  $\gamma$  counting. For the inhibition of scFv/folate by free folate, a nonsaturating concentration of scFv/folate (3 nM) that would generate maximal cytotoxicity was used together with various concentrations of free folate. Unless indicated otherwise, the specific release mediated by the folate conjugates was determined by subtracting the release in the absence of the conjugates (e.g. % specific release = {experimental counts - spontaneous counts}/{maximal counts - spontaneous counts} x 100).

## Results

**Characterization of scFv/Folate Conjugates.** The scFv of KJ16, an anti- $V\beta 8$  antibody, was purified from *E. coli* inclusion bodies after guanidine denaturation, refolding, and HPLC gel filtration. Purified scFv migrated as an apparent 35-kDa protein on SDS-PAGE gels (Figure 1A). Folate was coupled to the scFv using the carbodiimide

(EDC) reaction which links carboxyl groups of folate to primary amine groups on the protein. In engineering the scFv, the V<sub>L</sub> and V<sub>H</sub> domains were joined by the 26 residue linker, 205s, that contains 8 lysine residues (36, 44). We reasoned that the presence of multiple lysine residues in a highly accessible, solvent exposed region may result in higher folate densities in the linker region as opposed to the antibody V regions. In initial studies, folate was coupled to the scFv at a 100:1 folate to antibody molar ratio. Under these conditions, scFv/folate preparations contained an average of 3 to 8 folates per antibody (N = 3), based on spectrophotometric analysis. Consistent with this finding, migration of the scFv/folate conjugate on SDS-PAGE gels was slightly slower than scFv and the band was more diffuse (Figure 1A).

The folate density determined by photometry does not provide information about the heterogeneity of the conjugates. To determine the range of epitope densities within a single preparation, scFv/folate conjugates were examined by electrospray ionization mass spectrometry (Figure 1B). Unlabeled scFv demonstrated a single peak with a molecular mass of 29,082-Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular weight of a folate molecule (~400-Da), detectable up to 7 folates per antibody. Integration of mass spectra showed that greater than 85% of the scFv molecules were labeled with one or more folate molecules. Folate densities estimated from mass spectra were generally 1.5 to 2-fold lower than densities estimated by spectrophotometry. This could in part be due to lower solubility of high density conjugates under conditions required for mass spectrometry or to the dissociation of some folate molecules during ionization.

**Binding of scFv/Folate Conjugates to the T Cell Receptor.** To examine if folate conjugation affected the binding of scFv antibodies to the TCR, a scFv/folate conjugate was compared with unlabeled scFv. In a competitive flow cytometric assay, fluorescein labeled KJ16 Fab fragments were inhibited from binding the V $\beta$ 8-positive CTL clone 2C by unlabeled Fab fragments, scFv, or scFv/folate (Figure 2). As shown previously, scFv

antibodies have an approximate 3-fold higher apparent affinity than Fab fragments, possibly because of the presence of noncovalently associated scFv dimers (36). Comparison of folate-labeled and unlabeled scFv showed that the folate conjugate had an apparent affinity ~3-fold lower than scFv (i.e. approximately equal to KJ16 Fab fragments). This decreased binding, compared to unlabeled scFv, could be due either to chemical modification of active site residues with folate or to the interference of dimer formation by folate. The fact that folate conjugates bound the TCR as well as KJ16 Fab fragments, which have a  $K_D$  of ~130 nM (36, 45), indicated that the conjugates have potential to mediate lysis of target cells by CTLs.

#### **Binding of scFv/Folate Conjugates to Folate Receptors on Tumor Cells.**

The ability of scFv/folate conjugates to bind folate receptors (FR) on the surface of tumor cells was examined by a competition binding assay using  $^{125}\text{I}$ -labeled folate as the labeled ligand (Figure 3). The competition assay used the F2-MTX<sup>r</sup>A cell line that expresses the  $\beta$  isoform of the FR. Competitors included various concentrations of free folate, unlabeled scFv, and three different scFv/folate preparations. Examination of the binding curves showed that folate conjugated antibody, but not unlabeled scFv, binds to the FR<sup>+</sup> tumor cell line. However, based on molar folate concentration, the folate conjugates had a relative affinity that was approximately 10 to 30 fold less than free folate. This decrease in apparent affinity was consistent with previous observations (34) and could be partly attributed to the carbodiimide labeling procedure. This procedure links folate through either the  $\alpha$  or  $\gamma$  carboxyl groups but only linkage through the  $\gamma$ -carboxyl retains binding (46). It is also likely that some conjugated folates might be sterically hindered from interacting with a FR, either because they are too close to a neighboring folate or because of the location of nearby amino acid residues that prevent binding.

**CTL-Mediated Lysis of FR<sup>+</sup> Tumor Cells by scFv/Folate Conjugates.** The specificity and efficiency of tumor targeting with scFv/folate conjugates were examined in a  $^{51}\text{Cr}$  release assay with CTL clone 2C (Figure 4). Four different tumor cell lines that have

a range of cell surface FR densities were used as target cells: F2-MTX<sup>r</sup>A (200,000 sites/cell); La (60,000 sites/cell); L1210 (8,000 sites/cell); Mel (no-detectable FR). Each of the FR<sup>+</sup> cell lines was lysed in the presence of the scFv/folate conjugate and the extent of lysis was directly correlated with the expression level of the FR ( $r = 0.93$ ). Lysis was completely inhibited by free folate indicating that targeting of the tumor cells was specifically mediated by the folate receptor and not some other cell surface molecule. The lysis mediated by these conjugates was highly specific (e.g. the FR<sup>-</sup> cell line Mel was not lysed by the conjugate even at concentrations over  $10^5$ -times that required for detectable killing of the FR<sup>+</sup> cell line F2-MTX<sup>r</sup>A) and extremely potent (e.g. lysis was detectable at concentrations as low as 1 pM of scFv/folate). Thus, the presence of reduced folate carrier protein (as present in Mel, and in all other normal cells) does not result in cell destruction. It is important to point out that the FR density reportedly on ovarian tumors is even higher (~1 million/cell) than those on these tumor cell lines (47).

**Effects of Folate Density on Targeting.** To examine the effects of folate density and labeling on the targeting efficiency of scFv/folate conjugates, the antibody was labeled with folate under various carbodiimide-mediated coupling conditions. The carbodiimide EDC couples folate through the free carboxyl groups, but when used in the presence of protein it may also lead to protein modification and subsequent precipitation or inactivation. To evaluate the optimal levels of EDC for folate coupling, several different concentrations of EDC were used at a constant concentration of folate during coupling. Purified conjugates of scFv/folate prepared at three different EDC concentrations were examined in the cytotoxicity assay to determine the concentrations that yielded maximal specific targeting (Figure 5A). EDC used at the two lowest levels yielded conjugates with approximately equal targeting efficiency but higher levels yielded conjugates with reduced efficiency and frequently led to protein precipitation (Figure 5A and data not shown).

To directly examine the effect of folate density, activated folate was prepared at a constant EDC:folate ratio (5:1) and conjugates were produced by adding different ratios of

activated folate to the scFv protein. After dialysis to remove unreacted folate and excess EDC, conjugates were evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency. Folate densities ranged from approximately 1 to 20 folates per scFv. As shown in Figure 5B, each of the conjugates was capable of mediating lysis of the FR<sup>+</sup> tumor cell line by CTL clone 2C. However, conjugates with either the lowest density (1.3 folates/scFv) or highest density (20.4 folates/scFv) were 5 to 10-fold less effective than conjugates with intermediate folate densities. This experiment has been performed several times with similar results, indicating that the optimal folate density appears to be in the range of 5 to 15 folates per scFv, as determined by spectrophotometry. The reduction in targeting efficiency at higher folate densities is not due to the inability of these conjugates to bind to FR<sup>+</sup> cells (Figure 3), but is likely a consequence of chemical modification of amino acid residues important in scFv binding or stability.

**Comparison of Intact Antibody and scFv.** To determine the relative effectiveness of intact KJ16 IgG versus scFv-KJ16, both forms were labeled with folate at a 100:1 molar ratio of activated folate to antibody and under identical EDC reaction conditions (33 mM EDC, 6.7 mM folate). These conditions yielded folate densities of 7 and 5 for the scFv and intact antibody, respectively. Cytotoxicity assays with these preparations showed nearly identical targeting efficiencies for the intact and scFv forms of KJ16 (Figure 6). The concentration required to obtain 50% of the maximal specific release (EC<sub>50</sub>) was approximately 40 pM (1.2 ng/ml for scFv). Comparison of intact and scFv conjugates in an <sup>125</sup>I-folate binding assay indicated no significant difference in their ability to bind FR (data not shown).

Intact KJ16 antibody was also labeled at various folate densities to determine if targeting efficiency could be optimized further. Conjugates were again evaluated by spectrophotometry to determine folate densities and cytotoxicity assays to evaluate targeting efficiency (Figure 7). Folate densities ranged from approximately 4 to 126 folates per

antibody molecule. As shown with scFv preparations, high and low folate density resulted in decreased targeting effectiveness for the intact antibody. As with scFv preparations, the optimal densities appear to be in the range of 5 to 15 folates per antibody, as determined by spectrophotometry.

**Inhibition of scFv/Folate Conjugate-Mediated Lysis by Free Folate.** Normal serum folate may reduce the effectiveness of the scFv/folate conjugate by competing for the folate receptor *in vivo*. A  $^{51}\text{Cr}$  release assay was used to evaluate the effectiveness of the scFv/folate conjugate-mediated lysis at biologically relevant concentrations of free folate (Figure 8). Free folate was diluted in folate-free media and various concentrations were added to triplicate wells containing  $^{51}\text{Cr}$  labeled F2-MTX<sup>r</sup>A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (3 nM) that would generate maximal specific release. The resulting titration curve demonstrated that at normal human serum folate levels (9-36 nM, (48, 49)) the scFv/folate conjugate retained most of its activity. For instance, at 20nM folate, the scFv/folate conjugate exhibited over 80% of its CTL-mediated targeting potential. Although murine serum folate levels are significantly higher than normal human serum values (124 to 700 nM) due to folate-rich chow (50, 51)), even at these elevated folate concentrations, the scFv/folate conjugate exhibited 30 to 60% of its targeting potential.

## Discussion

The most effective agents for targeting solid tumors will likely have reduced sizes that allow greater tumor penetration. This report characterizes the smallest bispecific agent yet described for redirecting the activity of immune effector cells against tumors. Initial bispecific antibody studies to target ovarian tumors that express high affinity folate receptors have used intact heterobifunctional antibodies (~150 kDa) that bind to CD3 and the FR. These agents showed efficacy in animal models and they have recently entered testing in clinical trials (9, 11, 22). Although not yet reported for anti-FR antibodies,

several labs have shown that it is possible to engineer smaller bispecific antibodies of ~60 kDa by linking two scFv regions (52-54). Here we show that the size of a bispecific targeting agent can be reduced even further to ~30 kDa for the scFv/folate conjugates. Furthermore, the targeting efficiency of the engineered scFv/folate conjugate is comparable to that of the native intact antibody/folate conjugates.

For coupling of folate to the anti-V $\beta$ 8 scFv KJ16, a carbodiimide reaction that links the carboxyl groups of folate to the amino groups of the antibody was used. KJ16 scFv binds to the cell surface TCR with an affinity that is similar to the intact KJ16 antibody (36). The V<sub>L</sub> and V<sub>H</sub> regions of the scFv are linked by a 26 amino acid region that contains 8 lysine residues. We reasoned that this charged linker would be accessible to folate, would be located distal to the binding site, and would contain multiple attachment sites for folate through the EDC reaction. Under coupling conditions where folate concentrations are nonsaturating, the scFv and intact antibody have comparable densities of folate attached per molecule (~5 to 15 folates per molecule), despite the 5-fold greater size of the intact antibody. Cytolytic assays demonstrated that these conjugates have very similar targeting efficiencies (Figure 6).

Although folate is probably attached to lysines present in the linker, the scFv/folate conjugates are actually heterogeneous populations as evidenced by mass spectra (Figure 1B). Within a scFv/folate preparation there are most likely conjugates with enhanced targeting properties and conjugates with diminished targeting properties. The former could include not only those with folate at an accessible location, but perhaps those with multiple folates that allow for multivalent interactions with FRs on tumor cells. Conjugates which have diminished bispecific properties would include those that have folate attached through amine groups in the scFv active site or in regions which destabilize the V<sub>L</sub>-V<sub>H</sub> interaction. These are analogous to those preparations derived with very high folate densities, where the targeting efficiency is significantly reduced (Figures 5B and 7).



Several considerations suggest that further optimization of the scFv/folate conjugates could yield even more potent agents. The anti-TCR antibody KJ16 has a relatively modest  $K_D$  of  $\sim 100$  nM (36). Our lab is currently engineering higher affinity scFv antibodies by phage display; antibodies with  $K_D \leq 1$  nM have been routinely generated using this approach (55). In addition, the affinity of the scFv/folate conjugate for the FR<sup>+</sup> tumor cells was up to 30 fold less than the affinity of free folate for the FR (Figure 3). Coupling of folate through the  $\gamma$ -carboxyl, plus homogeneous linkage perhaps through cysteine or multiple cysteines, should improve the affinity for the FR. Alternatively, other folate analogs with higher affinity than folate could be employed (56). The use of these strategies should allow the development of scFv conjugates that have EC<sub>50</sub> values considerably less than those described in this report.

It is of significant note that the scFv agent described here is not only the smallest agent but is at least as potent *in vitro* as bispecific antibodies described in the literature. For example, the EC<sub>50</sub> of other bispecific agents range from 1 ng/ml to 100 ng/ml (47, 57-59). Various other antibody or folate-based targeting agents have EC<sub>50</sub> values that range from 0.1 ng/ml to 200 ng/ml (e.g. Refs. (43, 46, 60-63)). *In vitro* assays for these other agents typically involved 24 hour incubation periods while the cytotoxicity assays for CTL-mediated lysis, described here and elsewhere, are four hour incubations. Thus, there is reason to believe that the scFv/folate conjugate has considerable promise *in vivo*: It has an EC<sub>50</sub> of approximately 1 ng/ml, is smaller than the other agents, and remains effective at folate levels found in normal human serum.

The fact that the scFv/folate conjugate is in direct competition with serum folate brings about the intriguing possibility of modulating the effectiveness of scFv/folate conjugate treatment by altering the levels of serum folate. For instance, recent studies with mice have shown that serum folate can be intentionally decreased up to 100-fold with special low-folate diets (50, 51). The decreased folate concentration greatly enhanced the ability of a <sup>67</sup>Ga-labeled deferoxamine-folate conjugate to image FR<sup>+</sup> tumors *in vivo* (50). We

envision that similar low-folate diets will likewise enhance the therapeutic effectiveness of the scFv/folate conjugate. Conversely, serum folate could be increased in situations where non-specific T cell interactions lead to adverse side-effects. Thus, the use of folate as the small molecule ligand specific for a tumor antigen may allow for additional levels of regulation normally not available with other immunotargeting agents.

*In vivo* testing is currently underway in SV40 transgenic mice that develop choroid plexus tumors exhibiting elevated levels of the high affinity folate receptor (33). Preliminary results indicate that T cells specifically infiltrate the tumor after treatment with the scFv/folate conjugate (B.K.C., T.A.P., D.M.K., and E.J.R. unpublished data). At this time, it is unclear whether conjugate-bound-T cells extravasate into the tumor or if T cells first extravasate into the tumor and subsequently recognize the conjugate bound to cell surface FR. The latter mechanism would favor the enhanced tumor-penetration characteristics of the smaller scFv molecule and would likely lead to increased therapeutic effectiveness.

### Acknowledgments

We thank Drs. John Kappler and Philippa Marrack for providing the KJ16 hybridoma line. Carol Schlueter for providing the anti-L<sup>d</sup> ascites fluid and Drs. Brigle, Spinella, and Goldman for the DBA/2 tumor cell lines. Mass spectrometry was performed by Nelson Huang.

### References

1. Gorer, P. A. 1937. The genetic and antigenic basis of tumor transplantation. *Journal of Pathology and Bacteriology* 44:691.
2. Boon, T. 1993. Teaching the immune system to fight cancer. *Sci. Am.* 268:82.
3. Pardoll, D. M. 1994. A new look for the 1990s. *Nature* 369:357.

4. Klein, G. and Boon, T. 1993. Tumor immunology: present perspectives. *Curr. Opin. Immunol.* 5:687.
5. Fanger, M. W., Morganelli, P. M., and Guyre, P. M. 1992. Bispecific antibodies. *Crit. Rev. Immunol.* 12:101.
6. Liu, M. A., Nussbaum, S. R., and Eisen, H. N. 1988. Hormone conjugated with antibody to CD3 mediates cytotoxic T cell lysis of human melanoma cells. *Science* 239:395.
7. Nitta, T., Yagita, H., Sato, K., Okumura, K., and Ishii, S. 1990. Preliminary trials of specific targeting therapy against malignant glioma. *Lancet* 335:368.
8. Chen, J., Zhou, J. H., Mokotoff, M., Fanger, M. W., and Ball, E. D. 1995. Lysis of small cell carcinoma of the lung (SCCL) cells by cytokine-activated monocytes and natural killer cells in the presence of bispecific immunoconjugates containin a gastrin-releasing peptide (GRP) analog or a GRP antagonist. *J. Hematother.* 4:369.
9. Canevari, S., Stoter, G., Arienti, F., Bolis, G., Colnaghi, M. I., Di Re, E. M., Eggermont, A. M. M., Goey, S. H., Gratama, J. W., C.H.J., L., Nooy, M. A., Parmiani, G., Raspagliesi, F., Ravagnani, F., Scarfone, G., Trimbos, J. B., Warnaar, S. O., and Bolhuis, R. L. H. 1995. Regression of advanced ovarian carcinoma by intraperitoneal treatment with autologous T lymphocytes retargeted by a bispecific monoclonal antibody. *J. Nat. Cancer. Inst.* 87:1463.
10. Weiner, L. M., Clark, J. I., Davey, M., Li, W. S., Garcia de Palazzo, I., Ring, D. B., and Alpaugh, R. K. 1995. Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *Cancer Res* 55:4586.
11. Lamers, C. H., Gratama, J. W., Warnaar, S. O., Stoter, G., and Bolhuis, R. L. 1995. Inhibition of bispecific monoclonal antibody (bsAb)-targeted cytotoxicity by human anti-mouse antibodies in ovarian carcinoma patients treated with bsAb-targeted activated T-lymphocytes. *Int. J. Cancer* 60:450.

12. Coney, L. R., Tomassetti, A., Carayannopoulos, L., Frasca, V., Kamen, B. A., Colnaghi, M. I., and Zurawski, V. R., Jr. 1991. Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res.* 51:6125.
13. Buist, M. R., Molthoff, C. F. M., Kenemans, P., and Meijer, C. J. L. M. 1995. Distribution of OV-TL 3 and MOv18 in normal and malignant ovarian tissue. *J. Clin. Pathol.* 48:631.
14. Ross, J. F., Chaudhuri, P. K., and Ratnam, M. 1994. Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer* 73:2432.
15. Weitman, S. D., Frazier, K. M., and Kamen, B. A. 1994. The folate receptor in central nervous system malignancies of childhood. *J Neurooncol* 21:107.
16. Knapp, R. C. and Berkowitz, R. S. 1993. McGraw-Hill Inc, New York, p. 250.
17. Allen, J., Wisoff, J., Helson, L., Pearce, J., and Arenson, E. 1992. Choroid plexus carcinoma--responses to chemotherapy alone in newly diagnosed young children. *J Neurooncol* 12:69.
18. Dohrmann, G. J., Farwell, J. R., and Flannery, J. T. 1976. Ependymomas and ependymoblastomas in children. *J. Neurosurg.* 45:273.
19. Goldman, I. D. 1971. The characteristics of the membrane transport of amethopterin and the naturally occurring folates. *Ann. N.Y. Acad. Sci.* 186:400.
20. Westerhof, G. R., Jansen, G., van Emmerik, N., Kathmann, I., Rijksen, G., Jackman, A. L., and Schornagel, J. H. 1991. Membrane transport of natural folates and antifolate compounds in murine L1210 leukemia cells: role of carrier- and receptor-mediated transport systems. *Cancer Res.* 51:5507.
21. Huennekens, F. M., Vitols, K. S., Pope, L. E., and Fan, J. 1992. Membrane transport of folate compounds. *J. Nutr. Sci. Vitaminol. Spec No:*52-7.
22. Canevari, S., Miotti, S., Bottero, F., Valota, O., and Colnaghi, M. I. 1993. Ovarian carcinoma therapy with monoclonal antibodies. *Hybridoma* 12:501.

23. Coney, L. R., Mezzanzanica, D., Sanborn, D., Casalini, P., Colnaghi, M. I., and Zurawski, V. R. 1994. Chimeric murine human antibodies directed against folate binding receptor are efficient mediators of ovarian carcinoma cell killing. *Cancer Research* 54:2448.
24. van Ravenswaay Claasen, H. H., van de Griend, R. J., Mezzanzanica, D., Bolhuis, R. L., Warnaar, S. O., and Fleuren, G. J. 1993. Analysis of production, purification, and cytolytic potential of bi-specific antibodies reactive with ovarian-carcinoma-associated antigens and the T-cell antigen CD3. *Int. J. Cancer* 55:128.
25. Mezzanzanica, D., Garrido, M. A., Neblock, D. S., Daddona, P. E., Andrew, S. M., Zurawski, V. R., Jr., Segal, D. M., and Wunderlich, J. R. 1991. Human T-lymphocytes targeted against an established human ovarian carcinoma with a bispecific F(ab')<sub>2</sub> antibody prolong host survival in a murine xenograft model. *Cancer Res.* 51:5716.
26. Ferrini, S., Cambiaggi, A., Cantoni, C., Canevari, S., Mezzanzanica, D., Colnaghi, M. I., and Moretta, L. 1992. Targeting of T or NK lymphocytes against tumor cells by bispecific monoclonal antibodies: role of different triggering molecules. *Int. J. Cancer* 7:15.
27. Leamon, C. P. and Low, P. S. 1994. Selective targeting of malignant cells with cytotoxin-folate conjugates. *J Drug Target* 2:101.
28. Wang, S., Lee, R. J., Cauchon, G., Gorenstein, D. G., and Low, P. S. 1995. Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc. Natl. Acad. Asci. U.S.A.* 92:3318.
29. Brigle, K. E., Westin, E. H., Houghton, M. T., and Goldman, I. D. 1991. Characterization of two cDNAs encoding folate-binding proteins from L1210 murine leukemia cells. Increased expression associated with a genomic rearrangement. *J. Biol. Chem.* 266:17243.

30. Jansen, G., Schornagel, J. H., Westerhof, G. R., Rijksen, G., Newell, D. R., and Jackman, A. L. 1990. Multiple membrane transport systems for the uptake of folate-based thymidylate synthase inhibitors. *Cancer Res.* 50:7544.
31. Brigle, K. E., Spinella, M. J., Westin, E. H., and Goldman, I. D. 1994. Increased expression and characterization of two distinct folate binding proteins in murine erythroleukemia cells. *Biochem. Pharmacol.* 47:337.
32. Van Dyke, T. A., Finlay, C., Miller, D., Marks, J., Lozano, G., and Levine, A. J. 1987. Relationship between simian virus 40 large tumor antigen expression and tumor formation in transgenic mice. *J. Virol.* 61:2029.
33. Patrick, T. A., Kranz, D. M., Van Dyke, T. A., and Roy, E. J. 1996. Folate receptors as potential therapeutic targets in choroid plexus tumors of SV40 transgenic mice. *J. Neuro. Onc. In press.*
34. Kranz, D. M., Patrick, T. A., Brigle, K. E., Spinella, M. J., and Roy, E. J. 1995. Conjugates of folate and anti-T cell receptor antibodies specifically target folate-receptor-positive tumor cells for lysis. *Proc. Natl. Acad. Sci. USA* 92:9057.
35. Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 52:3402.
36. Cho, B. K., Schodin, B. A., and Kranz, D. M. 1995. Characterization of a single-chain antibody to the  $\beta$ -chain of the T cell receptor. *J. Biol. Chem.* 270:25819.
37. Friend, C., Patuleia, M. C., and De Harven, E. 1966. Erythrocytic maturation *in vitro* of murine (Friend) virus-induced leukemic cells. *NCI Monographs* 22:505.
38. Law, L. W., B, D. T., Boyle, P. J., and Miller, J. H. 1949. Observations on the effect of a folic-acid antagonist on transplantable lymphoid leukemias in mice. *J. Nat. Cancer Inst.* 10:179.
39. Brigle, K. E., Seither, R. L., Westin, E. H., and Goldman, I. D. 1994. Increased expression and genomic organization of a folate-binding protein homologous to the human

- placental isoform in L1210 murine leukemia cell lines with a defective reduced folate carrier. *J. Biol. Chem.* 269:4267.
40. Kranz, D. M., Sherman, D. H., Sitkovsky, M. V., Pasternack, M. S., and Eisen, H. N. 1984. Immunoprecipitation of cell surface structure of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc. Natl. Acad. Sci. USA* 81:573.
41. Haskins, K., Hannum, C., White, J., Rhoem, N., Kubo, R., Kappler, J., and Marrack, K. 1984. The antigen-specific major histocompatibility complex-restricted receptor on T cells. VI. An antibody to a receptor allotype. *J. Exp. Med.* 160:452.
42. Ozato, K., Hansen, T. H., and Sachs, D. H. 1980. Monoclonal antibodies to mouse MHC antigens. II. Antibodies to the H-2L<sup>d</sup> antigen, the product of a third polymorphic locus of the mouse major histocompatibility complex. *J. Immunol.* 125:2473.
43. Leamon, C. P. and Low, P. S. 1992. Cytotoxicity of momordin-folate conjugates in cultured human cells. *J. Biol. Chem.* 267:24966.
44. Whitlow, M. and Filpula, D. 1991. Single-chain Fv proteins and their fusion proteins. *Methods* 2:97.
45. Rojo, J. M. and Janeway, C. A., Jr. 1988. The biologic activity of anti-T cell receptor V region monoclonal antibodies is determined by the epitope recognized. *J. Immunol.* 140:1081.
46. Leamon, C. P., Pastan, I., and Low, P. S. 1993. Cytotoxicity of folate-*Pseudomonas* exotoxin conjugates toward tumor cells. Contribution of translocation domain. *J. Biol. Chem.* 268:24847.
47. Coney, L. R., Mezzanzanica, D., Sanborn, D., Casalini, P., Colnaghi, M. I., and Zurawski, V. R., Jr. 1994. Chimeric murine-human antibodies directed against folate binding receptor are efficient mediators of ovarian carcinoma cell killing. *Cancer Res.* 54:2448.
48. Kutsky, R. J. 1981. In *Handbook of Vitamins, Minerals, and Hormones, 2nd Edition*. Van Nostrand Reinhold, New York, p. 492.

49. Lentner, C. 1984. In *Geigy Scientific Tables, 8th revision, physical chemistry, composition of blood, hematology, somatometric data*. Vol. 3. Ciba-Geigy Limited, Basle, Switzerland, p. 131.
50. Mathias, C. J., Wang, S., Lee, R. J., Waters, D. J., Low, P. S., and Green, M. A. 1996. Tumor-selective radiopharmaceutical targeting via receptor-mediated endocytosis of gallium-67-deferoxamine-folate. *J. Nucl. Med.* 37:1003.
51. Gospe Jr., S. M., Gietzen, D. W., Summers, P. J., Lunetta, J. M., Miller, J. W., Selhub, J., Ellis, W. G., and Clifford, A. J. 1995. Behavioral and neurochemical changes in folate-deficient mice. *Physiol. Behav.* 58:935.
52. Gruber, M., Schodin, B., Wilson, E., and Kranz, D. M. 1994. Efficient Tumor Cell Lysis Mediated by a Bispecific Single-Chain Antibody Expressed in *E. coli*. *J. Immunol.* 152:5368.
53. Hollinger, P., Prospero, T., and Winter, G. 1993. "Diabodies": Small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA* 90:6444.
54. Kurucz, I., Titus, J. A., Jost, C. R., Jacobus, C. M., and Segal, D. M. 1995. Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J. Immunol* 154:4576.
55. Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. 1994. Making antibodies by phage display technology. *Annu. Rev. Immunol.* 12:433.
56. Elwood, P. C., Kane, M. A., Portillo, R. M., and Kolhouse, J. F. 1986. The isolation, characterization, and comparison of the membrane-associated and soluble folate-binding proteins from human KB cells. *J. Biol. Chem.* 261:15416.
57. Mack, M., Riethmuller, G., and Kufer, P. 1995. A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* 92:7021.
58. Zhu, Z., Zapata, G., Shalaby, R., Snedecor, B., Chen, H., and Carter, P. 1996. High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Biotech.* 14:192.



59. Sugiyama, Y., Aihara, M., Shibamori, M., Deguchi, K., Imagawa, K., Kikuchi, M., Momata, H., Azuma, T., Okada, H., Alper, Ö., Hitomi, J., and Yamaguchi, K. 1992. *In vitro* anti-tumor activity of anti-c-erbB-2 x anti-CD3ε bifunctional monoclonal antibody. *Jpn. J. Cancer Res.* 83:563.
60. Chaudhary, V. K., Queen, C., Junghans, R. P., Waldmann, T. A., FitzGerald, D. J., and Pastan, I. 1989. A recombinant immunotoxin consisting of two antibody variable domains fused to *Pseudomonas* exotoxin. *Nature* 339:394.
61. Chaudhary, V. K., Gallo, M. G., FitzGerald, D. J., and Pastan, I. 1990. A recombinant single-chain immunotoxin composed of anti-Tac variable regions and a truncated diphtheria toxin. *Proc. Natl. Acad. Sci. USA* 87:9491.
62. Burrows, F. J. and Thorpe, P. E. 1993. Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature. *Proc. Natl. Acad. Sci. USA* 90:8996.
63. Wels, W., Harwerth, I. M., Mueller, M., Groner, B., and Hynes, N. E. 1992. Selective inhibition of tumor cell growth by a recombinant single-chain antibody-toxin specific for the erbB-2 receptor. *Cancer Res* 52:6310.

## Footnotes

<sup>1</sup> This work was supported by grants from the University of Illinois Research Board (E.J.R.), the National Institutes of Health (AI35990, E.J.R. and D.M.K.), the Department of the Army (DAMD17-94-J-4347, D.M.K.) and the National Institutes of Mental Health (MH11189-01, T.A.P.) The ToFSpec mass spectrometer was purchased in part with Grant RR07141 from the Division of Research Resources, National Institutes of Health.

<sup>2</sup> To whom correspondence should be addressed: Department of Biochemistry, University of Illinois, 600 S. Matthews Ave., Urbana, IL 61801-3792.

<sup>3</sup> The abbreviations used are: CTL, cytotoxic T lymphocyte; FR, folate receptor; TCR, T cell receptor; scFv, single-chain antibody binding domain; MHC, major histocompatibility complex;  $K_D$ , dissociation constant; SV40, simian virus 40;  $EC_{50}$ , concentration of antibody-folate conjugate required for half-maximal specific release; fol, folate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; FITC, 5-aminofluorescein isothiocyanate; Ga, gallium; Fab, antigen binding fragment derived from papain digestion of Ig molecule; Fc, effector region of an antibody derived from papain digestion of Ig molecule; V regions, variable regions of IgG heavy and light chains;  $\epsilon$ , extinction coefficient; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, 10 mM phosphate buffer, 150 mM NaCl, pH 7.3; PBS-BSA, PBS containing 0.1% bovine serum albumin; MTX, methotrexate.

## Legends to Figures

**Figure 1. SDS-PAGE analysis and mass spectra of purified preparations of scFv KJ16 and scFv/folate conjugate.** *A*, Samples were electrophoresed through a 10% polyacrylamide gel under reducing and non-reducing conditions and proteins were visualized by staining with Coomassie Blue. *B*, Samples were concentrated, dialyzed against 1 mM potassium phosphate buffer, pH 8.0, and mass spectra were obtained on a ToFSpec using electrospray ionization. Purified scFv existed as a single species with molecular mass 29,082-Da. In contrast, folate-labeled scFv existed as a collection of antibody populations, each differing by the molecular weight of a folate molecule (~400-Da), detectable up to 7 folates per antibody.

**Figure 2. Binding of KJ16 scFv/folate to cell surface T cell receptor.** The binding of FITC-labeled KJ16 Fab fragments to the V $\beta$ 8-positive T cell clone 2C was inhibited by purified scFv/folate, scFv, or Fab fragments.  $6 \times 10^5$  2C cells were incubated 30 min at 4°C with FITC-labeled KJ16 Fab fragments and various concentrations of folate-labeled scFv KJ16 (▲), unlabeled scFv (■), and unlabeled Fab fragments (●). A relative affinity of the scFv/folate was determined by comparing the concentrations required to inhibit 50% of the FITC-labeled Fab fragments from binding the 2C TCR.

**Figure 3. Binding of KJ16 scFv/folate to folate receptors.**  $^{125}\text{I}$ -labeled folate was incubated with F2-MTX<sup>r</sup>A cells in the presence or absence of competitors for 1 h at 37°C. Concentrations refer to folate rather than antibody concentrations. Competitors included free folate (□) and KJ16 scFv/folate conjugates with different folate densities; 2.8 fol/scFv (○), 9.2 fol/scFv (■), and 20.4 fol/scFv (▲). Inhibition was not observed in the absence of competitor (Δ) or in the presence of unconjugated scFv (●) (error shown is  $\pm$  average SEM).

**Figure 4. Cytotoxicity assay of various tumor cells lines with the scFv/folate conjugate and CTL clone 2C.** Various concentrations of the scFv/folate conjugate were incubated with  $^{51}\text{Cr}$  labeled tumor cells and CTL 2C for 4 h at an effector-to target ratio of 5:1. Experiments were performed in the presence of anti- $\text{L}^{\text{d}}$  antibody to minimize lysis due to recognition of  $\text{L}^{\text{d}}$ , the nominal ligand for CTL 2C. Lysis correlated directly with the level of FR expressed by the cell line: F2-MTX $^{\text{r}}$ A (200,000 sites/cell,  $\blacktriangle$ ); La (60,000 sites/cell,  $\blacksquare$ ); L1210 (8,000 sites/cell,  $\bullet$ ); Mel (no-detectable FR,  $\circ$ ). Assays with free folate at a final concentration of 1.5  $\mu\text{M}$  were performed with the scFv/folate conjugate at a concentration of 0.09 nM (+ Folate).

**Figure 5. Effect of folate density on targeting with scFv/folate conjugates.**

A, To determine the EDC concentration that yielded the optimal targeting efficiency, three different EDC concentrations were evaluated ([EDC] = 13 mM,  $\bullet$ ; [EDC] = 65 mM,  $\blacksquare$ ; [EDC] = 260 mM,  $\blacktriangle$ ). Conjugates of scFv/folate were prepared at the indicated concentrations of EDC, at constant folate concentration, and used in cytotoxicity assays with the tumor cell line F2-MTX $^{\text{r}}$ A and CTL 2C. B, scFv/folate conjugates with different folate densities were prepared by labeling scFv protein with various amounts activated folate, using an EDC:folate ratio of 5:1 (EDC = 65 mM). These conjugates were tested in cytotoxicity assays using F2-MTX $^{\text{r}}$ A and CTL 2C. Folate densities were: 1.3 fol/scFv,  $\bullet$ ; 4.0 fol/scFv,  $\blacksquare$ ; 9.2 fol/scFv,  $\blacktriangle$ ; 9.3 fol/scFv,  $\circ$ ; 20.4 fol/scFv,  $\triangle$ .

**Figure 6. Comparison of scFv/folate and IgG/folate in CTL-mediated lysis of tumor cells.** Folate was conjugated to KJ16 scFv and intact antibody under identical conditions (3.3 mM EDC, 100:1 molar ratio of folate:antibody) yielding conjugates with 7 fol/scFv ( $\bullet$ ) and 5 fol/IgG ( $\blacksquare$ ). Cytotoxicity assays were performed with these conjugates using  $^{51}\text{Cr}$ -labeled F2-MTX $^{\text{r}}$ A cells and CTL clone 2C.

**Figure 7. Effect of folate density on targeting with IgG/folate conjugates.**

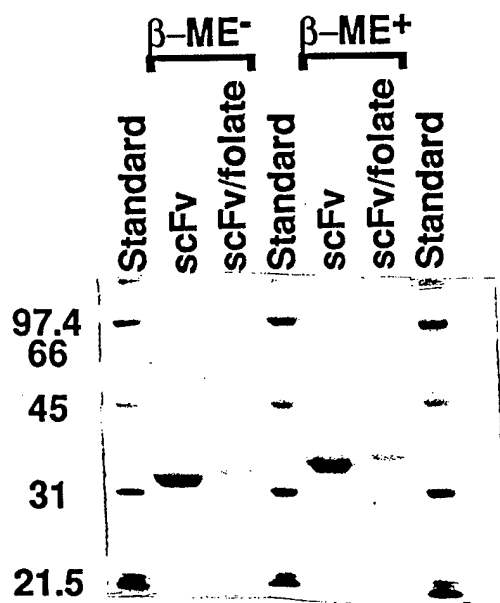
Intact antibody was labeled at several folate concentrations to determine if, like scFv, a specific range folate density would yield the optimal targeting effectiveness. Conjugates with the following densities: 4.5 fol/IgG, □ ; 7.0 fol/IgG, ● ; 13.6 fol/IgG, ▲ ; 56.7 fol/IgG, ■ ; and 126.2 fol/IgG, ○ were assayed with  $^{51}\text{Cr}$ -labeled F2-MTX<sup>r</sup>A cells and CTL clone 2C.

**Figure 8. Inhibition of scFv/folate-mediated lysis by free folate.**

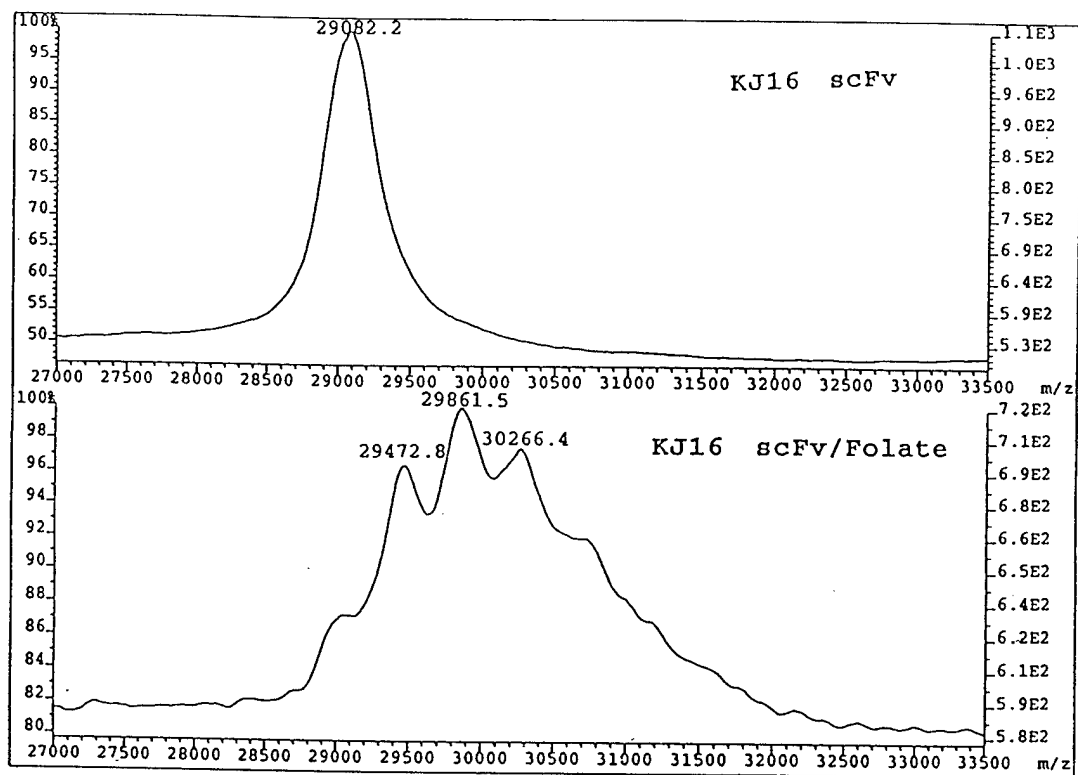
Various concentrations of free folate were added to triplicate wells containing  $^{51}\text{Cr}$  labeled F2-MTX<sup>r</sup>A cells, 2C effector cells, and a fixed, nonsaturating concentration of scFv/folate conjugate (folate density = 8) that would yield maximal specific release (3 nM). Inhibition was calculated as a function of the specific release in the absence of folate competitor.

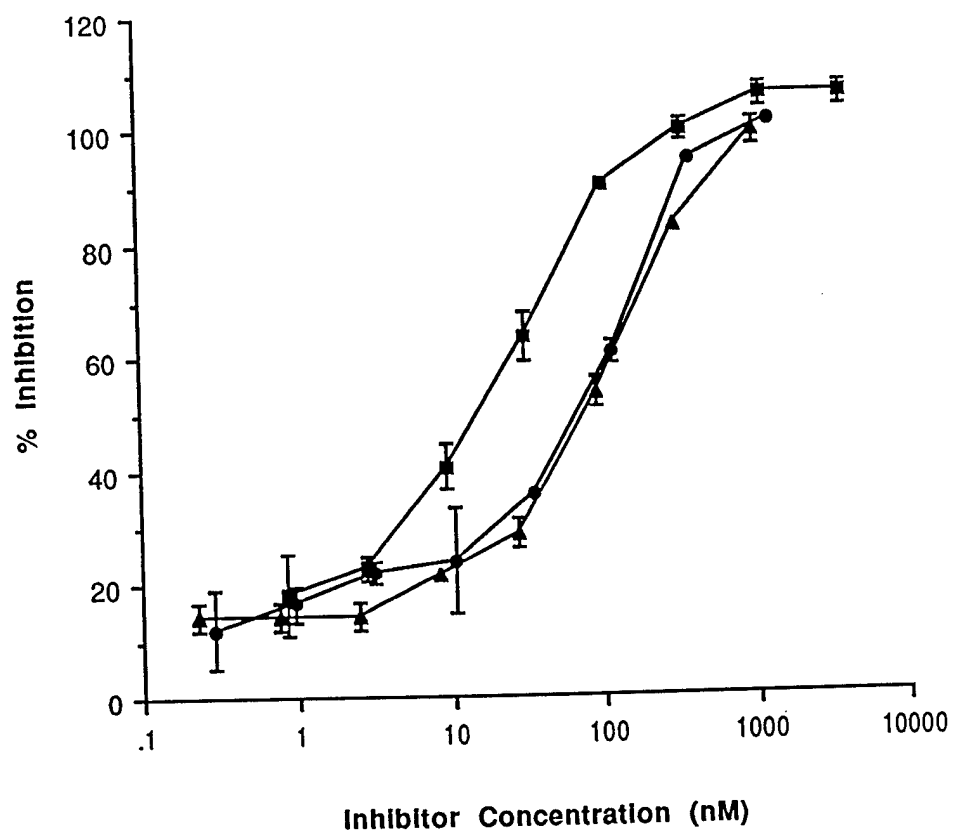
Normal human serum folate concentrations range from 9-36 nM (48, 49).

**A**

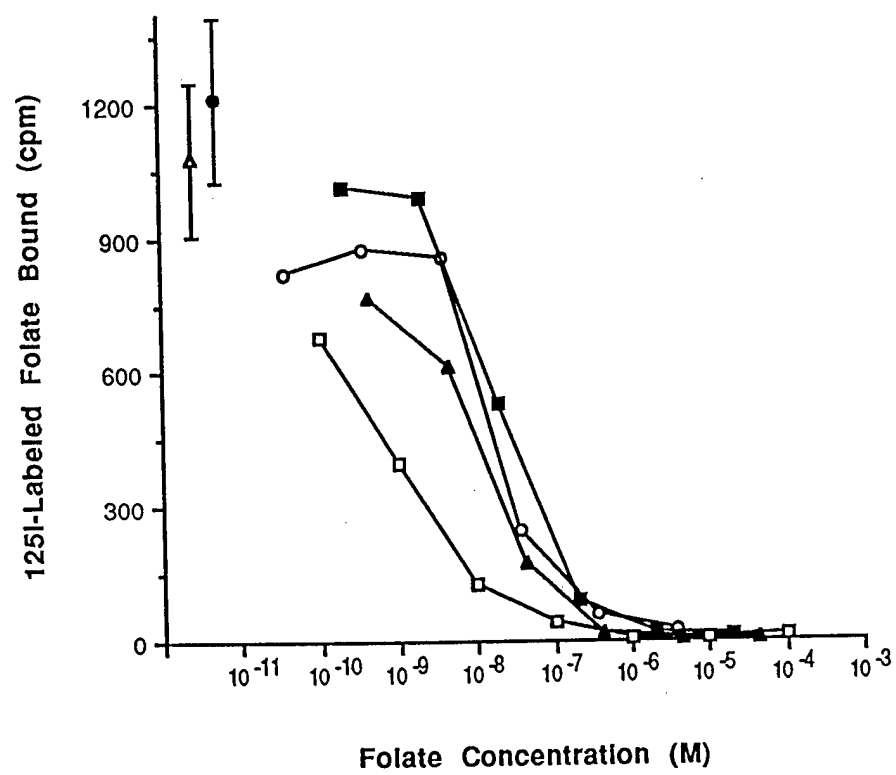


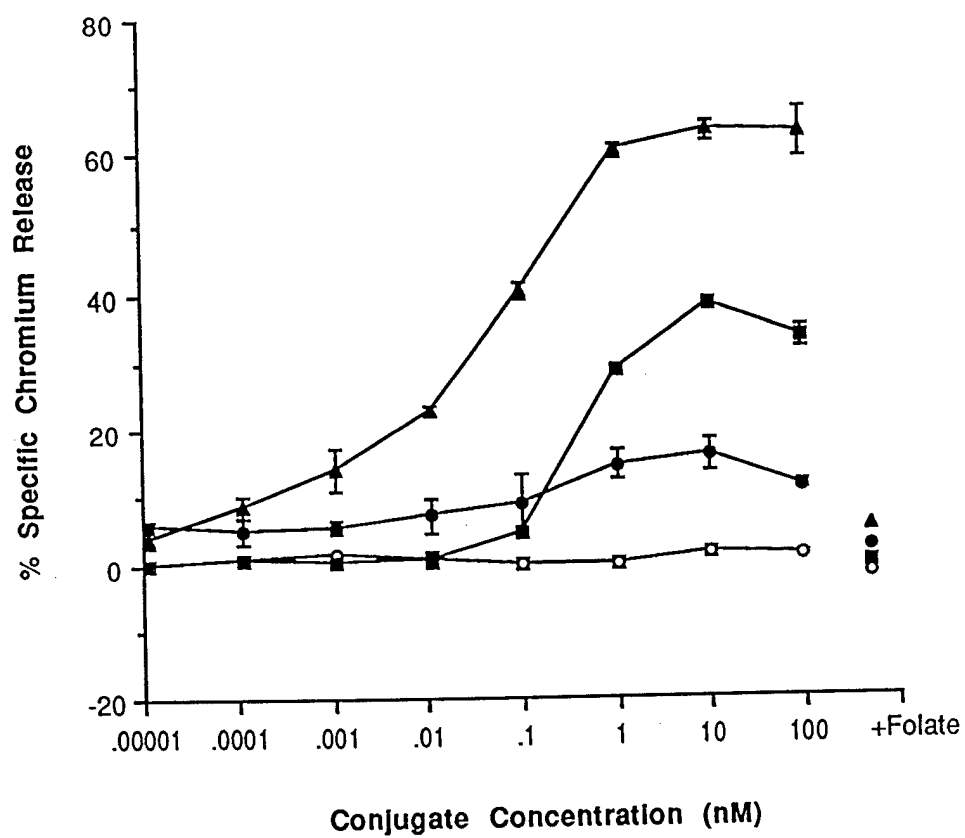
B



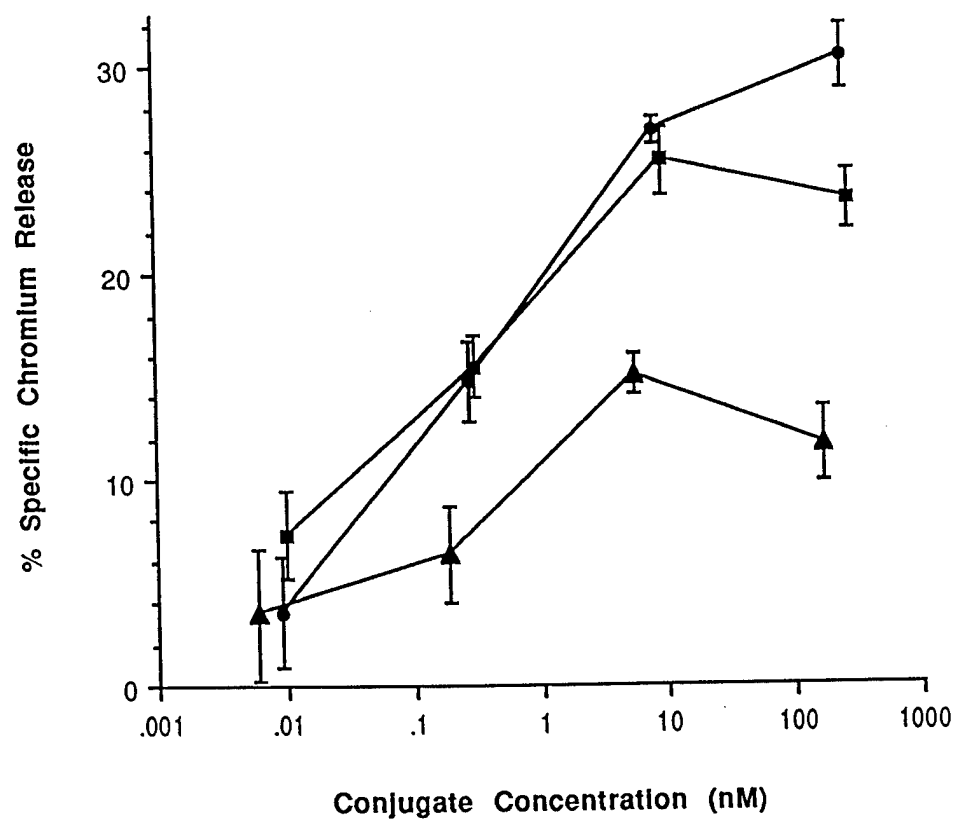




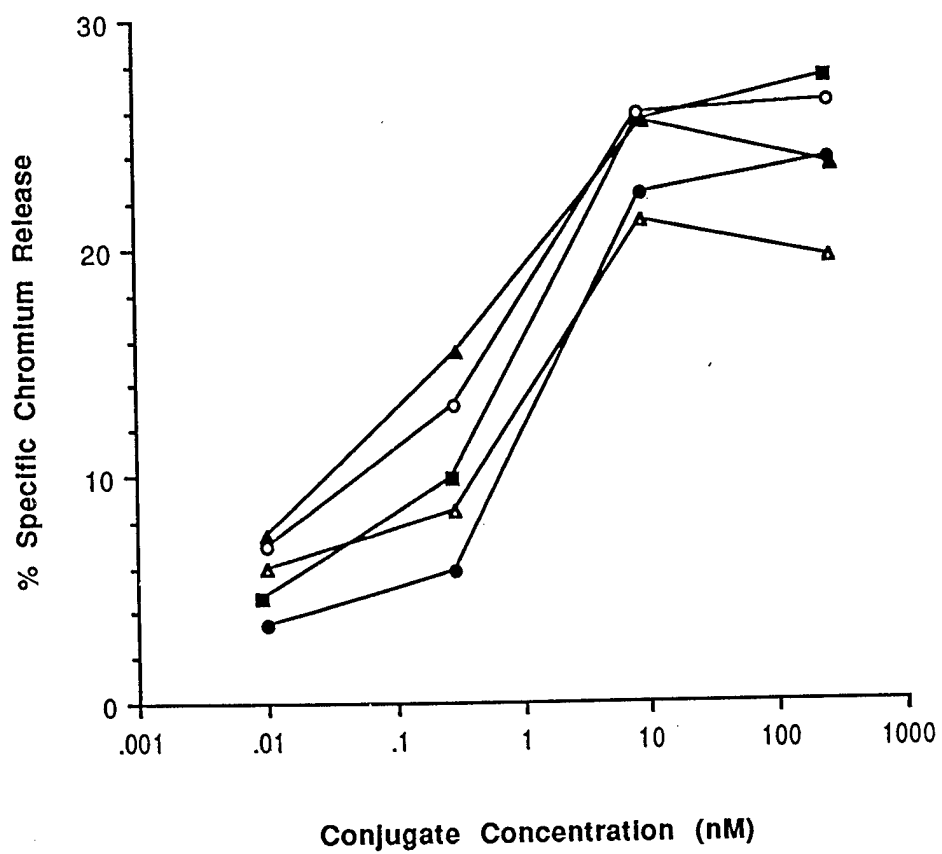


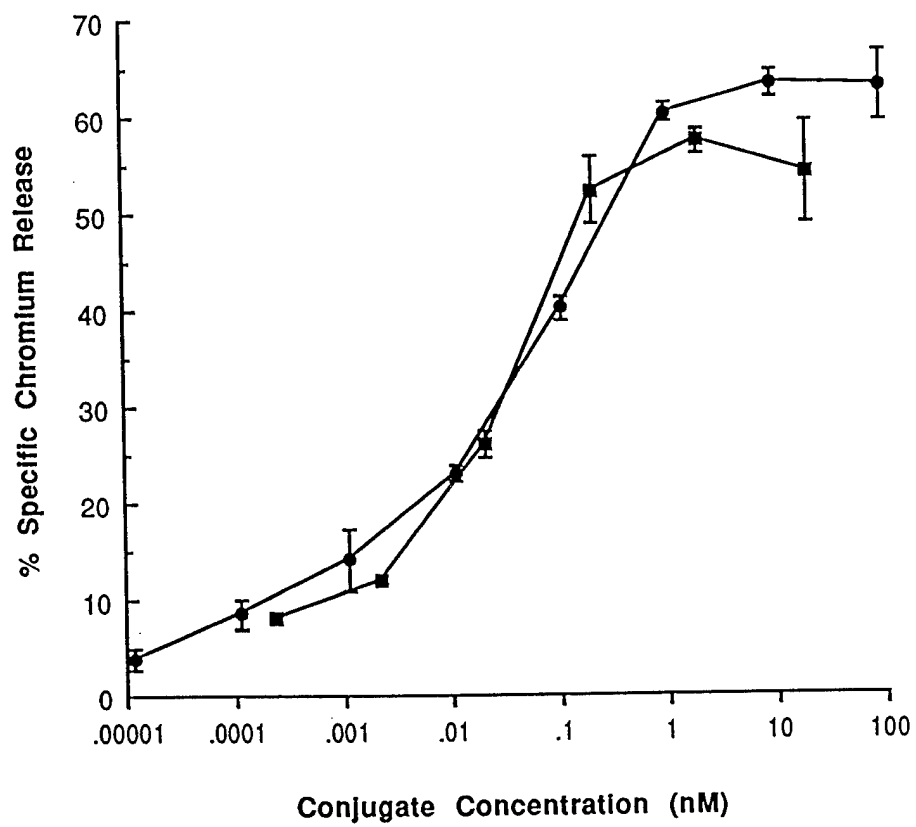


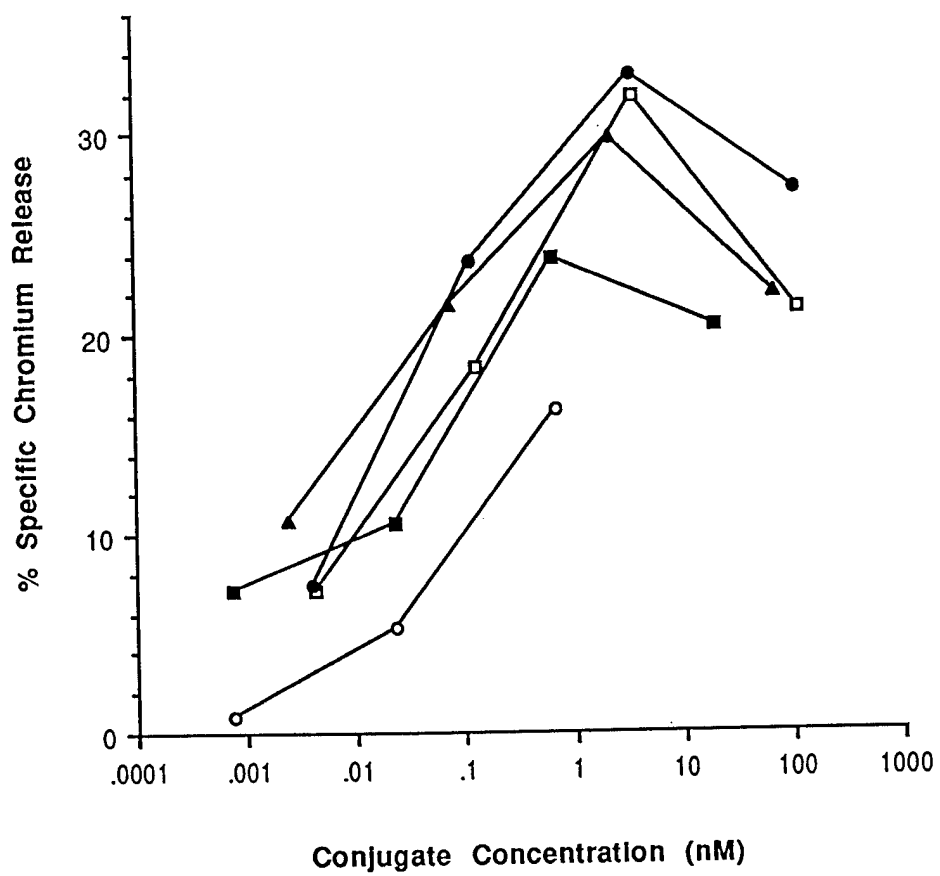
A

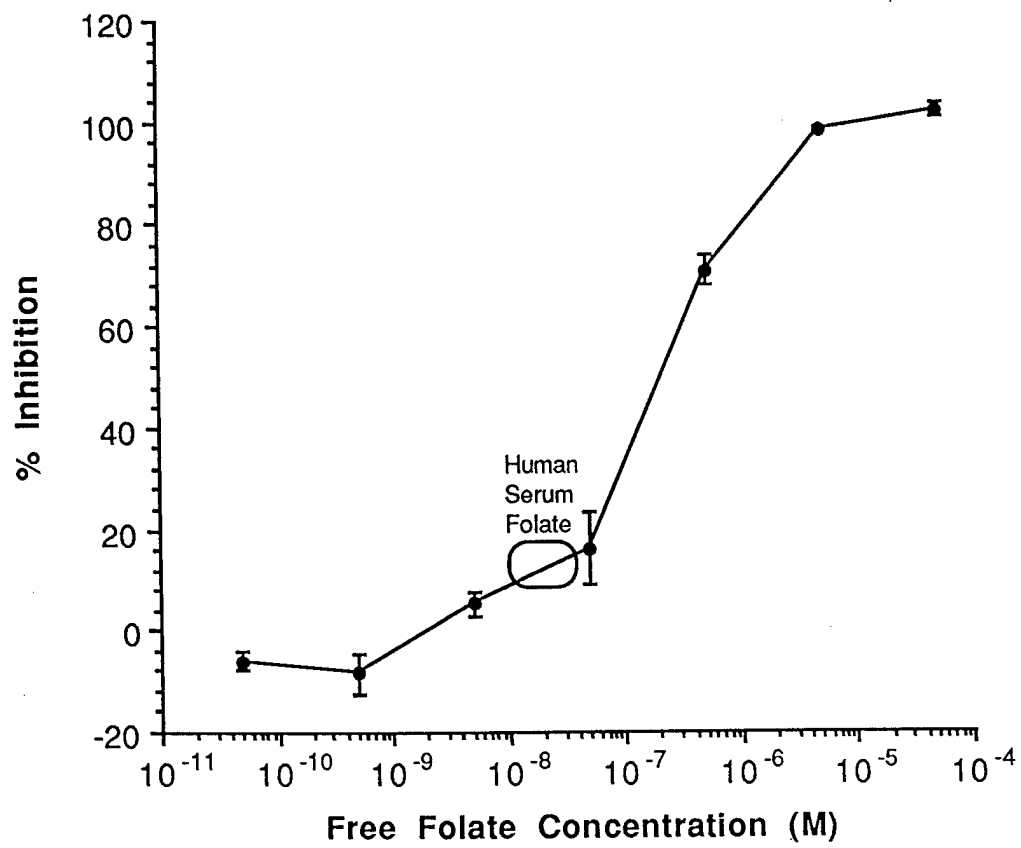


**B**









Lysis of Breast Cancer Cells Mediated by a Bispecific, Single-Chain  
Anti-erbB-2 Antibody

Meegan M. Gruber<sup>1</sup>, Pier G. Natali<sup>2</sup>, and David M. Kranz<sup>1</sup>

<sup>1</sup>Department of Biochemistry, University of Illinois, Urbana, IL 61801;  
and <sup>2</sup>Laboratory of Immunology, Regina Elena Cancer Institute,  
Rome, Italy

Short Title: Bispecific Single-Chain Anti-erbB-2 Antibody

Address correspondence to Dr. David M. Kranz, Department of  
Biochemistry, University of Illinois, Urbana, IL, 61801; (217)244-2821,  
*fax*(217)244-5858.



## **Abstract**

Bispecific antibodies that bind to erbB-2 and CD3 have been shown to mediate the lysis of erbB-2 positive breast cancer cells by cytotoxic T lymphocytes. Various forms of bispecific antibodies have been produced, but the form that will be most effective *in vivo* has yet to be determined. To begin to address this issue, we have engineered a bispecific single-chain antibody (scFv<sub>2</sub>) to erbB-2 and the mouse T cell receptor from the CTL clone 2C. This TCR has been previously expressed in transgenic mice and we have recently produced immunodeficient mice that express a monoclonal population of CTL with this receptor. In anticipation of targeting transplanted human tumors in these mice, the bispecific scFv<sub>2</sub> is characterized in this report. The erbB-2 specific monoclonal antibody 800E6 was cloned and expressed in *E. coli* as a 30kDa single chain Fv (scFv). Binding to erbB-2<sup>+</sup> SKBR-3 cells demonstrated that the affinity of the scFv for erbB-2 was identical to that of 800E6 Fab fragments (20nM). The bispecific scFv<sub>2</sub> 1B2/800 was constructed by joining anti-TCR scFv, 1B2, to the 800E6 scFv with a 25 residue linker. The 60kDa 1B2/800 scFv<sub>2</sub> bound to erbB-2 and could redirect lysis of erbB-2<sup>+</sup> BT-474 cells by the CTL clone 2C at concentrations of 1µg/ml and above. This antibody can now be tested in comparison with other engineered and conventional bispecific forms in the transgenic mouse model.

**Key Words:** Bispecific Antibody, erbB-2, Cytotoxic T Lymphocytes

## Introduction

Tumor-associated antigens present on the cell surface can serve as potential targets in the specific elimination of tumor cells. One such target is the oncogene product erbB-2 which is overexpressed in approximately 30% of human breast cancers(1). The efficacy of bispecific antibodies in redirecting cytotoxic T cell lysis of erbB-2<sup>+</sup> cells has been demonstrated in studies using anti-erbB-2 hybrid hybridomas and chemically coupled antibody fragments(2-5). Despite some successes of these bispecific antibodies in tumor therapy, the optimum method of preparing clinically useful antibodies has not been resolved. Each method has particular limitations, but in principle the optimal form will be the smallest possible agent with the highest affinities for both the tumor antigen and the T cell receptor. In addition, the yields of the final active agent must be sufficient to allow treatment in humans.

Recombinant technology and the generation of single chain fragments (scFv)(6-9) have led to the construction of single chain bispecific (scFv<sub>2</sub>) antibodies which may provide a more optimal method for preparing clinically useful reagents(10, 11). Production of the scFv<sub>2</sub> in *E.coli* provides a possible solution to problems of heterogeneity and yield, while the smaller size and lack of constant regions may increase tumor penetrance and decrease immunogenicity. Studies have demonstrated that the smaller scFv molecule can penetrate tumors more effectively than intact antibodies or Fab fragments, and they are retained longer in the tumor than in circulation(12). The latter property may also be important in terms of lower toxicity and immunogenicity.

Despite the progress on engineering various forms of bispecific antibodies, there has been little effort toward comparing their *in vivo* effects in an animal model. An ideal model would allow *in vivo* activation and targeting of transplanted human tumors with endogenous CTL. Toward this goal, we recently produced double transgenic mice that have the RAG-1 gene knocked out(13), and the TCR  $\alpha$  and  $\beta$  chain genes(14) from the CTL clone 2C (M. M. Gruber and D. M. Kranz, unpublished). These immunodeficient mice express a monoclonal population of CTL that do not recognize various human breast cancer lines.

To begin engineering bispecific antibodies to be tested in this model, the scFv of the anti-TCR antibody 1B2 (15, 16) was linked in this study to a scFv of the anti-erbB-2 antibody 800E6. The 800E6 antibody was previously shown to have a high affinity for the extracellular domain of erbB-2(17). We report here the characterization the 60kDa bispecific scFv<sub>2</sub> antibody that targets erbB-2 and mediates CTL 2C lysis of erbB-2 bearing tumor cells.

### **Materials and Methods**

**Cell Lines.** The BALB.B derived CTL clone 2C, which recognizes the alloantigen L<sup>d</sup>(18) and the superantigen SEB(19), was maintained in complete RPMI media [RPMI 1640 (Gibco, Grand Island, NY) containing 5mM HEPES (Sigma, St. Louis, MO), penicillin, streptomycin, 10% fetal bovine serum (Gibco), 1.3 mM L-glutamine (Gibco), 50 $\mu$ M 2-mercaptoethanol (Sigma)], and 10% supernatant from Con A-stimulated rat spleen cells as previously described(18). 2C cells were stimulated approximately every week with mitomycin C-treated BALB/c spleen cells. Daudi, a human

lymphoma that expresses class II HLA molecules, was maintained in complete RPMI media. Human breast carcinomas were obtained from the ATCC(Rockville, MD). SKBR-3 was maintained in McCoy's 5A medium containing 5mM HEPES, penicillin, streptomycin, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 $\mu$ M 2-mercaptoethanol. BT-474 was maintained in complete RPMI medium with 10 $\mu$ g/ml bovine insulin (Gibco). 800E6 is a BALB/c derived murine hybridoma which secretes an IgG1 monoclonal antibody that is specific for the erbB-2 transmembrane glycoprotein(17). 800E6 was maintained in complete RPMI medium.

**Preparation of Intact Antibodies and Fab Fragments.** Intact 800E6 was purified from ascites fluid by ammonium sulfate precipitation followed by DEAE column chromatography. Antibody was precipitated in 50% ammonium sulfate, the pellet resuspended in 50mM K<sub>2</sub>PO<sub>4</sub>, pH7.5, and then extensively dialyzed. A portion of intact antibody was dialyzed into TBS at ~2.2 mg/ml and Fab fragments were generated by digesting the antibody for 30 min in 2 U/ml papain (Sigma) at 37°C. Fab fragments were purified from residual intact antibody by size exclusion chromatography over a Superdex 200 column (Pharmacia, Piscataway, NJ).

**Construction and Expression of 800scFv.** Total RNA was isolated by the guanidinium thiocyanate-CsCl method. cDNA was generated by using approximately 5 $\mu$ g of the RNA with 35 units of reverse transcriptase (Promega, Madison, WI) and 5 $\mu$ M of oligoDT primer. Antibody V<sub>L</sub> and V<sub>H</sub> genes were cloned using the polymerase chain reaction (PCR) and degenerate primers for mouse antibodies: V<sub>H</sub>: 5'-

AAAGATGCATCCCAGGTGCAGCTGCAG(GC)AGTC(AT)GG(AG)  
(GC)(GC)G-3'; J<sub>H</sub>: 5'-

AAATAAGCTTTTGTCTGAGGAGACGGTGACTGAGGTTTCCT(GT)(G  
C)(AG)CCCCA-3'; V<sub>L</sub>: 5'-ATCGGACGTCGTGATGAC(CT)CAGTCTC-  
3'; J<sub>L</sub>: 5'-

TGATCCGCGGAGGAACGTTTTATTTCCAGCTTGGTCCC(ACT)(GC)  
C(AGT)CCGAA-3'. The V<sub>L</sub> and V<sub>H</sub> genes were inserted into a  
plasmid containing the 26 amino acid linker 205s(20). The  
construction included the ompA signal sequence and a 10 residue  
carboxy terminal tag derived from the *c-myc* protein. To construct the  
1B2/800 scFv2, 800E6 scFv was amplified by PCR with a 5' primer  
that included a Hind III site followed by a 205c'(7)linker gene (primer  
5'-

ATCTAAGCTTGCTAGCGCAGACGATGCCAAAAAAGACGCGGCGAA  
AAAAGACGATGCCAAAAAGGACGACGCCAAAAAAGATCTTGACGT  
CGTGATGACTCAGTCTCACAAATTCA-3') and a 3' primer to the  
plasmid (5'-GAACAAATCCAGATGGAGTTCTGA-3'). The modified  
800E6 scFv gene was inserted into a plasmid containing the 1B2  
scFv gene(16) The 1B2 scFv gene consists of the ompA signal  
sequence, the 1B2 V<sub>L</sub> gene, a 42-base pair linker gene [called  
212,(19)], the 1B2 V<sub>H</sub> gene, and a 30-base pair gene encoding the *c-  
myc* peptide(21). The modified 800E6 scFv was inserted at the Hind  
III site between the 1B2 scFv V<sub>H</sub> gene and the *c-myc* gene. All  
primers were synthesized by the Genetic Engineering Facility at the  
University of Illinois Biotechnology Center. Both 800E6 scFv and  
1B2/800 scFv2 were expressed, as previously described(22), using a  
hybrid O<sub>L</sub>/P<sub>R</sub> I phage promoter in an *E.coli* strain that contains a

temperature sensitive C1857 repressor gene. Induction, solubilization, and purification were performed as previously described(22). Briefly, small scale cultures or fermentations were grown to an A<sub>600</sub> of 1.0 at 30°C, at which point recombinant protein expression was induced by temperature shift to 42°C for 1 hr. Cells were disrupted by passage through a microfluidizer, centrifuged at low speed to pellet unlysed cells, and the supernatant centrifuged to pellet inclusion bodies. Inclusion bodies were solubilized for 12-24 hours in 6 M guanidine hydrochloride (10 ml guanidine per cell pellet equivalent obtained from 1 liter of culture).

**Purification of scFv and scFv<sub>2</sub> Fragments.** 800E6 scFv was refolded by dialysis at ~1mg/ml against 0.1M Tris, pH 8.0, 2mM EDTA, pH 8.0, 0.4M arginine (TEA buffer) at 4°C. Refolded scFv was centrifuged at 15,000xg to remove precipitate and injected onto a Superdex 200 size exclusion column pre-equilibrated in phosphate buffered saline, pH 7.3. 1B2/800 scFv<sub>2</sub> was applied to a Superdex 200 size exclusion column pre-equilibrated in 4M guanidine-HCl, 50mM Tris, pH8.0, 2mM EDTA to remove protein still aggregated in the presence of denaturant. Fractions corresponding to the monomeric peak were adjusted to ~1mg/ml in 4M guanidine buffer and refolded by dialysis in TEA buffer. To isolate monomeric 1B2/800, refolded scFv<sub>2</sub> was applied to a Superdex 200 size exclusion column pre-equilibrated in PBS, pH7.3. All columns were run at 0.5ml/min and eluents monitored by absorbance at 280nm.

**Binding Assays.** To determine the intrinsic affinity of the 800E6 Fab fragments, a saturation binding assay was performed with <sup>125</sup>I-labeled Fab fragments. Increasing concentration of labeled 800E6

Fab fragments were added to wells containing  $2 \times 10^5$  SKBR-3 to a total volume of 75  $\mu$ l. After a 60 minute incubation on ice, 50  $\mu$ l was layered over 300  $\mu$ l of 20% olive oil, 80% dibutylphthalate in an elongate microcentrifuge tube and flash spun to pellet cells. Tubes were frozen on dry ice/ethanol and cut to isolate the cell pellet from the solution. Fractions were monitored in a  $\gamma$ -counter and a Scatchard plot was generated to determine  $K_D$  of the Fab fragments. To detect scFv and scFv2 binding to erbB-2 on the surface of breast cancer cells, a competition assay was performed with  $^{125}$ I-800E6 Fab fragments. Various concentrations of unlabeled 800E6 Fab fragments, scFv, or scFv2 were added, together with a constant amount (200,000 cpm) of  $^{125}$ I-labeled 800E6 Fab fragments, to tubes containing  $2 \times 10^5$  SKBR-3 or BT-474 cells in a total volume of 75  $\mu$ l. Fractions were monitored in a  $\gamma$ -counter and inhibition curves were prepared for unlabeled 800E6 Fab fragments, scFv, and scFv2 antibodies.

**Cytotoxicity Assays.** Target cells were harvested by trypsinization, washed with RPMI medium, and incubated with 50  $\mu$ l of  $^{51}$ Cr (2.5mCi/ml) for 1 hr at 37°C. After washing with RPMI media, cells were added to round bottom wells of a 96-well plate at  $2 \times 10^4$  cells/well. 1B2/800 scFv2 was added at various concentrations, and 2C was added at  $2 \times 10^5$  cells/well. Assays were incubated for 4 hrs at 37°C in a 5%CO<sub>2</sub> incubator. Supernatants were removed and monitored with a  $\gamma$ -counter. Specific  $^{51}$ Cr-release was determined as follows: %Specific  $^{51}$ Cr release = [(experimental counts - spontaneous counts)/(maximal counts - spontaneous counts)]x100.

## Results

**Cloning and construction of 800E6 scFv.** PCR of cDNA from anti-erbB-2 hybridoma 800E6 was performed using degenerate primers for mouse V<sub>H</sub> and V<sub>K</sub>. Amplified product of the expected sizes (350-400 bp) were obtained for each set of primers (data not shown). The V<sub>H</sub> product was cloned into a vector flanked at the 5' end by the sequence for a 26 amino acid linker, 205s(20) and at the 3' end by the sequence for a 10 residue peptide derived from the *c-myc* protein. This *c-myc* tail was inserted at the carboxy terminus in order to allow detection with the anti-*c-myc* monoclonal antibody, CT14-G4.3(23). The V<sub>K</sub> gene was then cloned upstream of the V<sub>H</sub> between the ompA signal sequence and the 205s linker (Figure 1A).

The nucleotide and predicted amino acid sequences of 800E6 scFv are shown in Figure 1B. Both the V<sub>H</sub> and V<sub>L</sub> genes contained the typical invariant residues present in Ig domains, including two appropriately spaced cys residues and the GXGT motif within the J regions. The calculated molecular mass of the mature protein is 28,772 daltons.

**Expression and Purification of 800E6 scFv.** Previous studies from our lab showed that single chain antibodies were expressed in the insoluble fraction of the lysed *E. coli* cell pellet(10, 16, 20). Thus, inclusion body pellets from cells that contained the 800E6 scFv gene were solubilized in guanidine and dialyzed against a Tris-arginine buffer to allow refolding(24). SDS-PAGE analysis showed that approximately 80% of the refolded inclusion body protein migrated at 30kDa (Figure 2). 800E6 scFv was purified further through a non-



denaturing Superdex 200 size exclusion column (see SDS-PAGE gel, Figure 2).

**Binding Properties of 800E6 scFv.** The intrinsic affinity of the 800E6 Fab fragment was determined by saturation binding of  $^{125}\text{I}$ -Fab fragments to SKBR-3. The  $K_D$  was determined by Scatchard analysis to be 20nM (data not shown). To determine the relative affinity of 800 scFv for erbB-2, a competition assay was performed with  $^{125}\text{I}$ -labeled Fab fragments of 800E6 and unlabeled Fab fragments or HPLC purified scFv. As shown in Figure 3, scFv bound to erbB-2 on SKBR-3 cells at nearly equivalent concentrations as Fab fragments.

**Construction of anti-TCR/anti-erbB-2 bispecific scFv2.**

Bispecific single-chain (scFv2) 1B2/800, with binding domains of both the anti-TCR antibody 1B2 and the anti-erbB-2 antibody 800E6, was constructed as shown in Figure 4. 1B2 is a clonotypic antibody that reacts with the TCR from the BALB.B CTL clone 2C(15). The 1B2 scFv construction contained the  $V_L$  and  $V_H$  chain genes, intra-antibody linker, 212, and a 10 residue *c-myc* tail at the carboxy terminus. To construct the scFv2, the 800E6 scFv was amplified by PCR using a 5' primer which contained the sequence for the 205c' linker, and a 3' primer that included a HindIII site within the *c-myc* region for cloning into the 1B2 scFv vector. The linker/800E6 scFv fragment was cloned between the 1B2 $V_H$  and the *c-myc* tail as shown in Figure 4.

**Expression and Purification of anti-TCR/anti-erbB-2 scFv2.**

Inclusion body pellets from cells that contained the scFv2 gene were solubilized in 6M guanidine. To separate monomeric scFv2 from

proteins which remained aggregated in the presence of guanidine, the solubilized inclusion body was subjected to Superdex 200 size exclusion in 4M guanidine. The 60kDa peak fractions were each refolded by dialysis into Tris-Arginine buffer, and analyzed by SDS-PAGE (data not shown). Fractions containing the 60kDa protein were pooled, dialysed against buffer, and subjected to non-denaturing size exclusion chromatography. The peak that eluted at the approximate size of monomeric scFv2 (~60kDa) migrated as two distinct species under reducing conditions (data not shown) and non-reducing conditions (Figure 5). The second protein may be a contaminant from *E. coli*, or a differentially processed form of the scFv2 (with or without the signal sequence). Although we have not examined these possibilities directly, we have observed that the binding activity of the HPLC purified 1B2/800 scFv2 (see below) is equal to that of a histidine-tagged version of 1B2/800 (1B2/800-6histidine) that was purified on an immobilized metal affinity column (M. M. Gruber, and D. M. Kranz, unpublished). This suggests that the HPLC purified material contained approximately the same fraction of properly refolded scFv2 as the histidine-tag purified protein.

**Binding Properties of anti-TCR/anti-erbB-2 scFv2.** To examine binding of the scFv2 to erbB-2 on the surface of human breast cancer cells, a competition assay was performed with <sup>125</sup>I-labeled 800E6 Fab fragments. As shown in Figure 6, crude as well as partially purified preparations of scFv2 were able to bind erbB-2 on BT-474 cells. In contrast to the monovalent 800E6 scFv which bound erbB-2 at nearly equimolar concentrations as 800E6 Fab fragments, bispecific antibody preparations were 10 to 25-fold less efficient than

unlabeled Fab fragments. A possible explanation for the observed reduction in binding is that a significant fraction of the scFv2 preparation is not properly folded.

### **Bispecific Antibody Activity in CTL-Mediated Killing**

**Assays.** As the scFv2 had erbB-2 binding activity, it was of interest to examine if the antibody could redirect CTL 2C to lyse target cells. As expected, the L<sup>d</sup> alloreactive CTL 2C was unable to lyse human, erbB-2 positive breast carcinoma line BT-474 without the bispecific antibody. However, BT-474 were lysed when 1B2/800 scFv2 was added to the CTL/target cell assay (Table 1). The observed lysis was dependent on the concentration of bispecific antibody (Figure 7) although the maximal lysis obtained with BT-474 cells never reached more than ~25% specific release. The reason for this is unknown. As expected, scFv2 mediated lysis could be inhibited with monospecific 1B2 or 800 intact Ab (Table 1, exp. 1). The lysis required covalent linkage of the two antibody binding domains, as evidenced by the inability of a mixture of 1B2 and 800E6 monospecific antibodies to redirect lysis (Table 1).

Since anti-TCR antibodies also have the potential to suppress T cell responses, we tested whether concentrations of scFv2 that led to lysis were inhibitory in another cytolytic assay. In this case, the human lymphoblastoid line, Daudi, was incubated with the superantigen SEB which is recognized by CTL 2C(23). This recognition is inhibited at 1B2 concentrations that are several fold lower than inhibition of the peptide/L<sup>d</sup> ligand recognized by 2C(16). The 1B2/800 scFv2, however, did not inhibit the recognition at concentrations of 25µg/ml (Table 1). Thus, it should be possible to

use concentrations of bispecific antibody that are effective at mediating killing but will not inhibit normal TCR-mediated responses. However, this result also suggests that the 1B2 domain of the scFv<sub>2</sub>, like the 800E6 domain, is not folded in a large fraction of the protein.

### Discussion

Bispecific antibodies that bind to erbB-2 on breast cancer lines have been used *in vitro* and *in vivo* in T cell mediated therapy(3, 4, 25-28). *In vivo* mouse models using bispecific antibodies against human erbB-2<sup>+</sup> xenografts have demonstrated that redirected T cell lysis leads to decreased tumor size and prolonged survival(4). In these studies, both the bispecific agent and *in vitro* activated human T cells were administered to the mice receiving the transplanted tumors. Although clinical trials have begun to examine the efficacy of the first generation of bispecific antibodies, F(ab')<sub>2</sub> fragments, recent advances in genetic engineering have allowed various smaller and potentially less immunogenic forms to be produced. These forms have included non-covalently associated scFv and Fab fragments, and covalently linked scFv<sub>2</sub>.

To identify the forms that will have the highest probability of success in patients, there are significant issues that need to be addressed in an appropriate animal model. We believe that the animal model should allow the *in vivo* activation of endogenous T cells in order to resemble the situation to be encountered in humans. Transplantation of human tumors into mice has made this scenario impossible because xenotransplants are rejected by immunocompetent mice. To circumvent this problem we have produced an immunocompromised strain of mouse that contains a

monoclonal population of T cells that express the TCR from the CTL 2C. In this report, we have characterized a bispecific single-chain antibody that can redirect lysis by the CTL clone 2C.

The anti-erbB-2 antibody 800E6 and the anti-TCR antibody 1B2 were chosen because they have high affinities. Monomeric 800E6 scFv also retained high affinity after refolding from guanidine solubilized inclusion bodies. Based on these results, greater than 90% of the scFv preparation appeared to be folded properly. In contrast, after refolding the bispecific scFv<sub>2</sub> was at least 10-fold less effective than the scFv in inhibiting binding by 800E6 Fab fragments. Although not formally proven, we believe that the presence of other V regions (i.e. 1B2 V<sub>H</sub> and V<sub>L</sub>) lead to misfolding of the final scFv<sub>2</sub> protein. Nevertheless, the scFv<sub>2</sub> protein was active at mediating cytolysis of the human tumor cell line BT-474 in the  $\mu\text{g/ml}$  (10-100nM) range. This finding indicates that the 25 amino acid residue linker that joins the two scFv has sufficient flexibility to allow CTL and breast cancer cells to be bridged and to activate the CTL.

Based on the 10 to 100-fold greater activity reported for several conventional bispecific antibodies, it is likely that the folding of scFv<sub>2</sub> preparations will need to be improved in order to optimize these reagents for *in vivo* use(28, 29). Alternatively, other methods of creating linked scFv domains may be useful(30, 31) and these forms can be prepared using the 1B2 and 800E6 scFv genes in comparison with the scFv<sub>2</sub> described here. Finally, the finding that the scFv<sub>2</sub> 1B2/800 mediated lysis of erbB-2<sup>+</sup> human tumor cells by the mouse CTL 2C suggests that these agents can now also be compared in the *in vivo* model described above.

### Acknowledgements

This work was supported by grant DAMD-17-94-J-4347 from the Department of the Army(to DMK). MMG was generously supported by a research scholarship from the Carle Cancer Center. PGN was supported by PF, CNR, ACRO and by the Italian Ministry of Public Health.

### References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 1987;235:177-82.
2. Carter P, Rodriguez ML, Lewis GD, Figari I, Shalaby MR. Towards an immunotherapy for p185HER2 overexpressing tumors. In: Ceriani RL, ed. *Breast cancer research and immunology*. New York: Plenum, 1994;83-94. (Antigen and antibody molecular engineering in breast cancer diagnosis and treatment).
3. Shalaby MR, Shepard HM, Presta L, *et al*. Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J Exp Med*.1992;175: 217-25.
4. Weiner LM, Holmes M, Adams GP, LaCreta F, Watts P, Garcia de Palazzo I. A human tumor xenograft model of therapy with a bispecific monoclonal antibody targeting c-erbB-2 and CD16. *Cancer Res*. 1993;53:94-100.

5. Weiner LM, Holmes M, Richeson A, *et al.* Binding and cytotoxicity characteristics of the bispecific murine monoclonal antibody 2B1. *J Immunol.* 1993;151:2877-86.
6. Bird RE, Hardman KD, Jacobson JW, *et al.* Single-chain antigen-binding proteins. *Science.* 1988; 242:423-426.
7. Huston JS, Levinson D, Mudgett-Hunter M, *et al.* Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl Acad Sci USA.* 1988; 85:5879-83.
8. Wright A, Shin SU, Morrison SL. Genetically engineered antibodies: progress and prospects. *Crit Rev Immunol.* 1992;12:125-68.
9. Pluckthun A. Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immunol Rev.* 1992;130:151-63.
10. Gruber M, Schodin BA, Wilson ER, Kranz DM. Efficient tumor cell lysis mediated by a bispecific single chain antibody expressed in *E. coli*. *J Immunol.* 1994;152:5368-74.
11. Mack M, Riethmuller G, Kufer P. A small bispecific antibody construct expressed as a functional single-chain molecule with high tumor cell cytotoxicity. *Proc Natl Acad Sci USA.* 1995;92:7021-5.
12. Yokota T, Milenic DE, Whitlow M, Schlom J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res.* 1992;52:3402-8.

13. Mombaerts P, Iacomini J, Johnson RJ, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 1992;68:869-77.
14. Sha WC, Nelson CA, Newberry RD, Kranz DM, Russel JH, Loh DY. Selective expression of an antigen receptor on CD8-bearing T lymphocytes in transgenic mice. *Nature*. 1988;335:271-274.
15. Kranz DM, Tonegawa S, Eisen HN. Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc Natl Acad Sci USA*. 1984;81:7922-6.
16. Schodin BA, Kranz DM. Binding affinity and inhibitory properties of a single-chain anti-T cell receptor antibody. *J Biol Chem*. 1993;268:25722-7.
17. Digiesi G, Giacomini P, Fraioli R, *et al*. Production and characterization of murine mAbs to the extracellular domain of human neu oncogene Product GP185<sup>HER2</sup>. *Hybridoma*. 1992;11:519-27.
18. Kranz DM, Sherman DH, Sitkovsky MV, Pasternack MS, Eisen HN. Immunoprecipitation of cell surface structure of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc Natl Acad Sci USA*. 1984;81:573-7.
19. Soo Hoo WF, Kranz DM. Role of CD8 in Staphylococcal enterotoxin B-mediated lysis by cytotoxic T lymphocytes. *J Immunol*. 1993;150:4331-7.



20. Cho BK, Schodin BA, Kranz DM. Characterization of a single-chain antibody to the  $\beta$ -chain of the T cell receptor. *J Biol Chem*. 1995;270:25819-26.
21. Evan GI, Lewis GK, Ramsay G, Bishop JM. Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol Cell Bio*. 1985;5:3610-17.
22. SooHoo WF, Lacy MJ, Denzin LK, *et al*. Characterization of a single-chain T cell receptor expressed in *E. coli*. *Proc Natl Acad Sci USA*. 1992;89:4759-63.
23. Pantoliano MW, Bird RE, Johnson S, *et al*. Conformational stability, folding, and ligand-binding affinity of single-chain Fv immunoglobulin fragments expressed in *Escherichia coli*. *Biochem*. 1991;30:10117-25.
24. Buchner J, Rudolph R. Renaturation, purification, and characterization of recombinant Fab-fragments produced in *Escherichia coli*. *Biotech*. 1991;9:157-62.
25. Sugiyama Y, Aihara M, Shibamori M, *et al*. *In vitro* anti-tumor activity of anti-c-erbB-2xanti-CD3 $\epsilon$  bifunctional monoclonal antibody. *Jpn J Cancer Res*. 1992;83:563-7.
26. Tsukamoto H, Nakamura Y, Masuko T, *et al*. Specific targeting of in vitro-activated antitumour effector cells using anti-CD3xanti-c-erbB-2 bispecific antibody. *Immunol Cell Bio*. 1993;71:109-15.
27. Weiner LM, Clark JI, Davey M, *et al*. Phase I trial of 2B1, a bispecific monoclonal antibody targeting c-erbB-2 and Fc gamma RIII. *Cancer Res*. 1995;55:4586-93.

28. Zhu Z, Lewis GD, Carter P. Engineering high affinity humanized anti-p185HER2/anti-CD3 bispecific F(ab')<sub>2</sub> for efficient lysis of p185HER2 overexpressing tumor cells. *Int J Cancer* 1995;62:319-24.
29. Kurucz I, Titus JA, Jost CR, Jacobus CM, Segal DM. Retargeting of CTL by an efficiently refolded bispecific single-chain Fv dimer produced in bacteria. *J Immunol.* 1995;154:4576-82.
30. Hollinger P, Prospero T, Winter G. "Diabodies": Small bivalent and bispecific antibody fragments. *Proc Natl Acad Sci USA.* 1993;90:6444-8.
31. Kostelny SA, Cole MS, Tso JY. Formation of a bispecific antibody by the use of leucine zippers. *J Immunol.* 1992;148:1547-53.

## Figure Legend

### Figure 1. Schematic of the anti-erbB-2 single-chain

**antibody 800E6. A.** cDNA was generated from the 800E6 hybridoma, and the  $V_L$  and  $V_H$  genes were amplified by PCR using a set of degenerate primers. The genes were linked by a sequence encoding a 26-amino acid linker called 205s, and the construct was cloned behind the ompA signal sequence and included a carboxyl-terminal peptide from the *c-myc* protein. **B.** Sequence of scFv 800E6 derived from the 800E6 hybridoma, and sequence for *c-myc* tail.

### Figure 2. SDS-PAGE analysis of purified 800E6 scFv.

Single chain antibodies were prepared as described in Materials and Methods. Crude scFv represents the TEA-dialyzed preparation before size exclusion chromatography, and purified scFv represents fractions from the Superdex 200 column. Molecular mass markers (in kDa) are shown. Proteins were visualized by staining with Coomassie blue.

**Figure 3. Binding of scFv to erbB-2 on SKBR-3.** Various concentrations of unlabeled 800E6 Fab fragments or scFv were added, together with a constant amount (200,000cpm) of  $^{125}\text{I}$ -labeled 800E6 Fab fragments, to wells containing  $2 \times 10^5$  SKBR-3 cells. After 1 hr on ice, cells were pelleted through a mixture of olive oil and dibutylphthalate, and monitored on a gamma counter. Percent inhibition was determined relative to binding of  $^{125}\text{I}$ -labeled 800E6 Fabs in the absence of inhibitor.

**Figure 4. Schematic of bispecific single-chain anti-T cell receptor, anti-erbB-2 antibody scFv2.** 1B2  $V_L$  and  $V_H$  genes, linked by a sequence encoding the 14 amino acid 212 linker (GSTSGSGKSSEGKG), and 800E6  $V_L$  and  $V_H$  genes linked by a

sequence encoding the 26 amino acid 205s linker (SSADDAKKDAAKKDDAKKDDAKKDAS) were joined using overlapping primers in the polymerase chain reaction. The monospecific single-chain constructs were linked by a gene that encodes a 25 amino acid linker called 205c' (ASADDAKKDAAKKDDAKKDDAKKDL). The bifunctional single-chain was cloned between the ompA signal sequence (MKKTAIAIAVALAGFATVAQA), and a 10 amino acid peptide from the *c-myc* protein (EQKLISEEDL).

**Figure 5. Non-denaturing gel filtration profile and SDS-PAGE analysis of purified scFv<sub>2</sub>.** Crude scFv<sub>2</sub> was applied to a Superdex 200 size exclusion chromatography column in 6M guanidine. The 60kDa peak was dialyzed against TEA, pH 8.0 and applied to a Superdex 200 HPLC column in PBS, pH 7.3. Absorbance was monitored at 280nm. The column was calibrated with molecular mass standards, and a 60kDa peak corresponding to the monomeric scFv<sub>2</sub> was identified. **Insert. Non-reducing SDS-PAGE of gel filtration HPLC fractions.** All single chain antibodies were prepared as described in the text. HPLC fractions represent 60kDa peak fractions as labeled on the HPLC profile. Molecular mass markers (in kDa) are shown. Proteins were visualized by silver stain technique.

**Figure 6. Binding of scFv<sub>2</sub> to erbB-2 on BT-474 cells.**

Various concentrations of 800E6 Fab fragments or scFv<sub>2</sub> were added, together with a constant amount (200,000 cpm) of <sup>125</sup>I-labeled 800E6 Fab fragments, to wells containing 2x10<sup>5</sup> BT-474 cells. Crude scFv<sub>2</sub> represents the TEA dialyzed preparation, before HPLC

purification. scFv2 was purified by passage through Superdex 200 in 6M guanidine. After 1 hr on ice, cells were pelleted through a mixture of olive oil and dibutylphthalate, and monitored on a gamma counter. Percentage inhibition was determined relative to binding of  $^{125}\text{I}$ -labeled 800E6 Fabs in the absence of inhibitor.

**Figure 7. Bispecific scFv2 1B2/800 mediates lysis of erbB-2<sup>+</sup> human breast cancer cells by CTL 2C.** BT-474 cells were incubated with 50  $\mu\text{l}$  of  $^{51}\text{Cr}$  (2.5mCi/ml) for 1 hr at 37°C. After washing with PBS, target cells were plated at  $2 \times 10^4$  cells/well. The scFv2 was added at various concentrations, and CTL 2C was added at  $2 \times 10^5$  cells/well. Plates were incubated for 4 hr at 37°C, then supernatants were removed and monitored with a gamma counter.

Table 1. Tumor cell lysis mediated by scFv2 and CTL clone 2C

Experiment 1	<u>antibody</u>	<u>μg/ml</u>	<u>Inhibitor</u>	<u>% Specific 51Cr Release</u>
				<u>BT-474</u>
	-	-	-	0
	1B2 Ig + 800E6 Ig	25 + 25	-	4
	scFv2	13	-	22
	scFv2	13	25 μg/ml 1B2 Ig	0
	scFv2	13	25 μg/ml 800E6 Ig	0
	scFv2	13	-	0*
Experiment 2	<u>antibody</u>	<u>μg/ml</u>		<u>% Specific 51Cr Release</u>
				<u>BT-474</u>
	scFv2	5		20
	scFv2	2.5		21
	scFv2	1.3		18
	scFv2	0.6		6
Experiment 3	<u>antibody</u>	<u>μg/ml</u>		<u>% Specific 51Cr Release</u>
				<u>Daudi/SEB</u>
	-	-		64
	scFv2	25		61

\*In this assay, target cells were added to wells with media and scFv2, without effector cells. In all other wells 2C CTLs were added at an effector to target cell ratio of 10:1.

**A**



**B**

GACGTGCTGATGACTCAGTCTCACAATTCATGTCCACATCAGTAGGAGACAGGGTCAGCATCACTGCAAG  
 DVVMTQSHKFMSTSVGDRVSI TCK  
 GCCAGTCAGGATGTTGGGTACTGCTGTAGCCTGGTATCAACAGAAACCAGGGCAATCTCCTAACTACTGATT  
 ASQDVGTA VAWYQQKPGQSPKLLI  
 TACTGGGCATCCACCCGGCACACTGGAGTCCCTGATCCCTTCACAGGCAGTGGATCTGGGACAGATTTCACT  
 YWASTRHTGTGVPDRFTGSGSGTDF  
 CTCACCATTAGCAATGTGCAGTCTGAAGACTTGGCAGATTATTTCTGTACGAATATAGCAGTTATGCGACG  
 L T I S N V Q S E D L A D Y F C Q Q Y S S Y R T  
 TTCGGTGCAGGGACCAAGCTGGAATTAAGCT  
 FGAGTKLEIKR

**800E6 V<sub>L</sub>**

TCCTCCGGGATGATGCTAAGAAGGATGCTGCTAAGAAGGATGATGCTAAGAAGATGATGCTAAGAAGAT  
 SSADDAKKDA A K K D D A K K D D A K K D  
 GCATCC  
 A S

**205s linker**

CAGGTGCAGCTGCAGCAGTCAAGGGGAGAGCTGATGAAGCCTGGGGCTCAGTGAAGATATCTGCAAGGCT  
 QVQLQQSGGELMKPGASVKISCKA  
 ACTGGCTACACATTCAGTAACTACTGGATAGAGTGGGTGAAGCAGAGGCTGGACATGGCTTGAGTGGATT  
 TGYTFSNYWI EWVKQRP GHGLEWI  
 GGAGAGATTTTACTGGAAGTGGTAGTACTAATACAGTGAAGCTCAAGGGCAAGGCCACATTCAGTCA  
 GEILPGSGSTN YNEKLKGKATFT A  
 GACACATCTCCACACAGCCTACATGCAACTCAGCAGCCTGACATCTGAGGACTCTGGGCTCATTTACTGT  
 D T S S N T A Y M Q L S S L T S E D S G V Y Y C  
 GCAAGAGGGGGGTAACTACCCGTACTACTTTGACTACTGGGGCCAGGAACCTCAGTCAACCGTCTCTCA  
 A R G G G N Y P Y Y F D Y W G P G T S V T V S S

**800E6 V<sub>H</sub>**

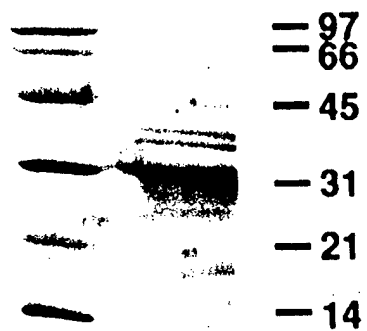
GAACAAAGCTTATTTCCGAAGAAGATTG  
 EQKLISEEDL

**c-myc tail**

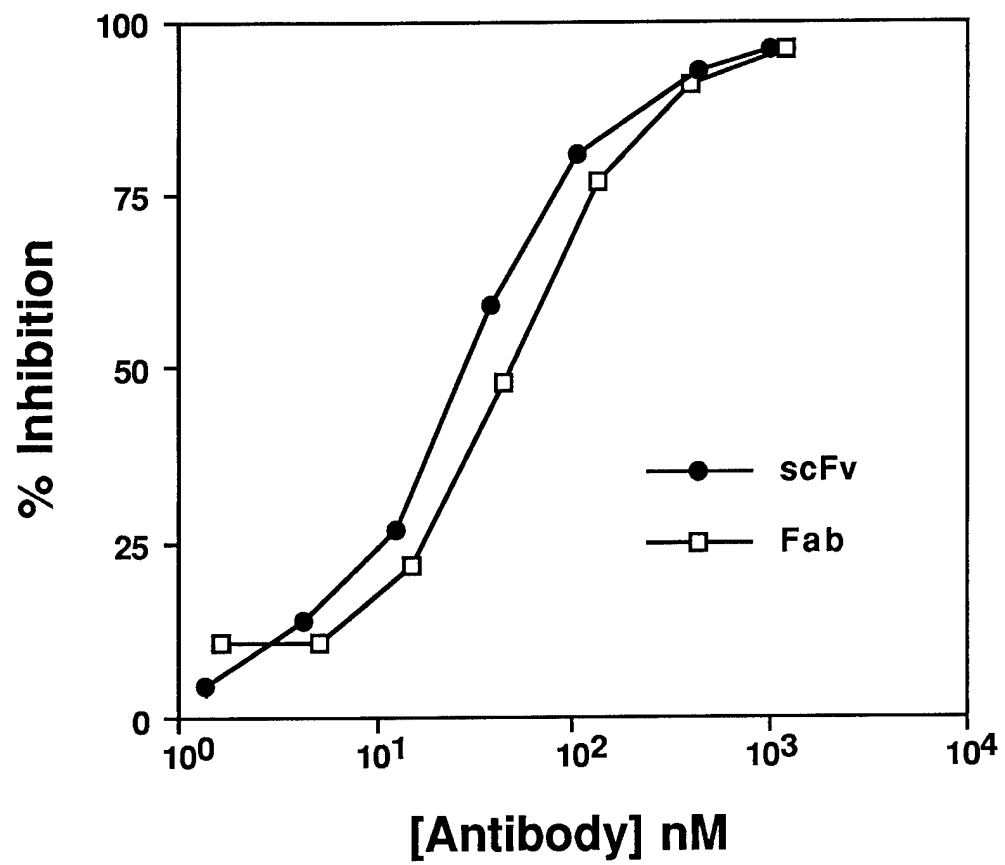
**purified  
scFv**



**crude  
MW scFv**

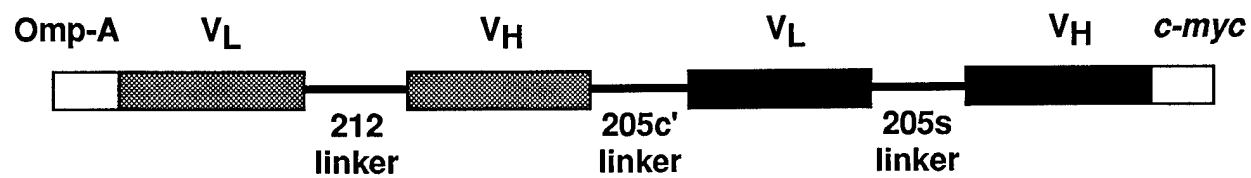


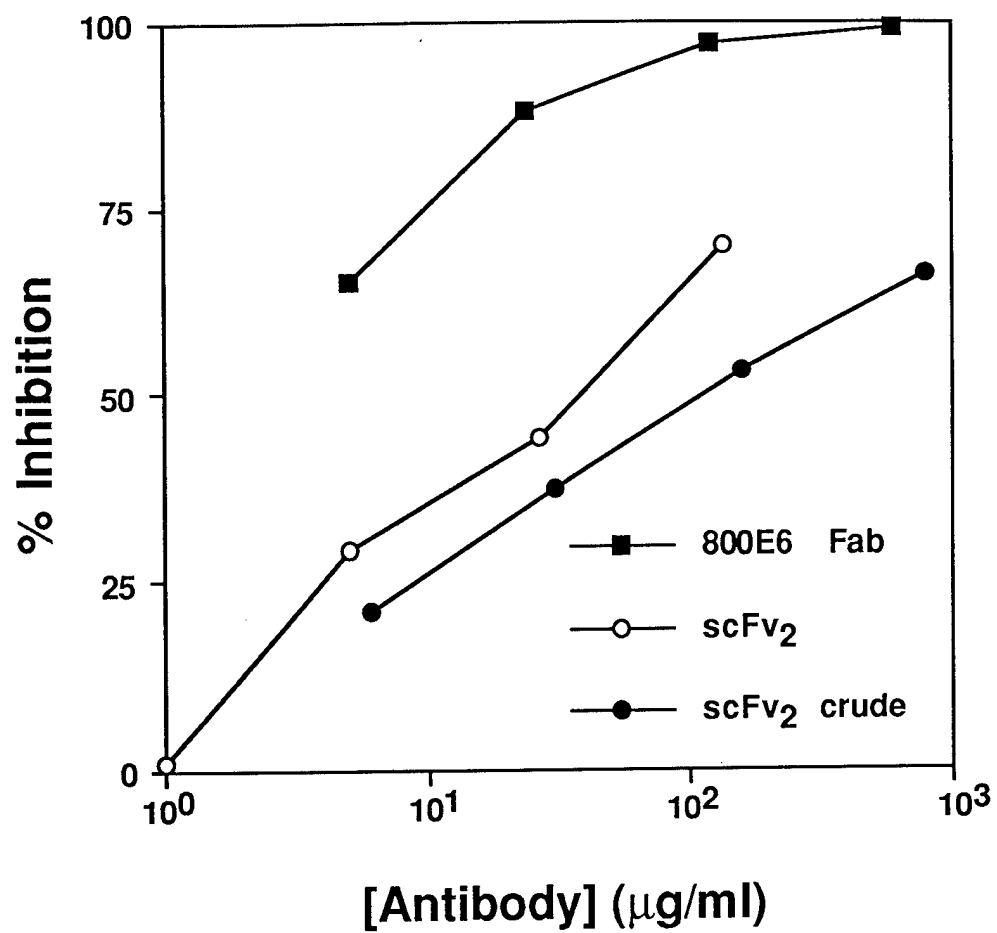


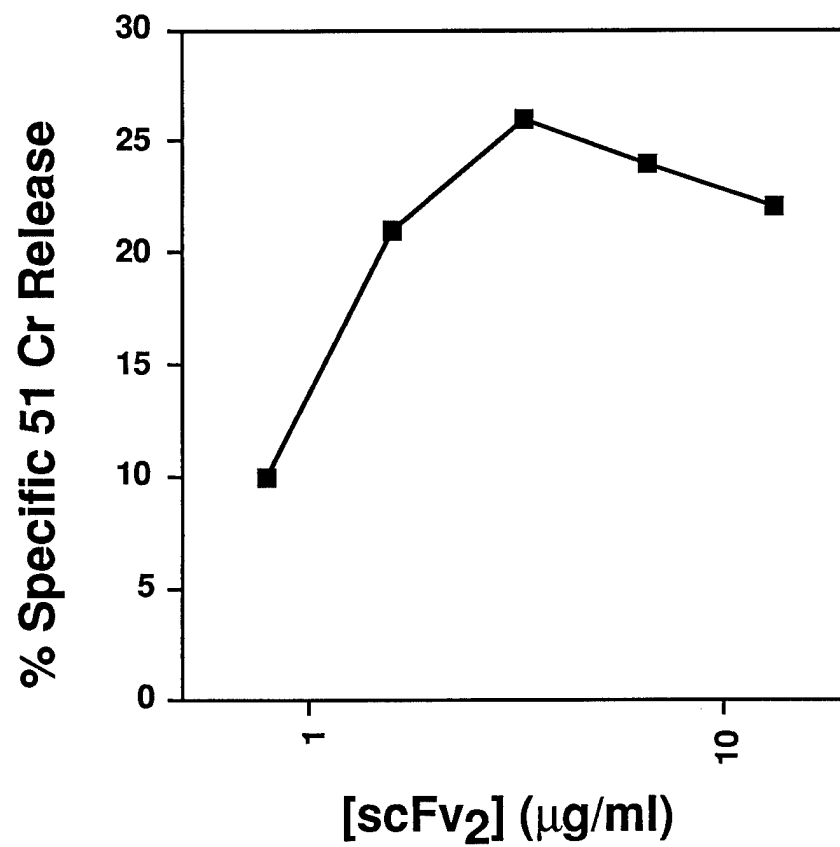


**1B2 (Anti-T Cell Receptor)**

**800 (Anti-erbB-2)**







A Combination of IFN- $\gamma$  and TNF- $\alpha$  Can Increase the Susceptibility of erbB-2  
Positive Tumors to CTL-Mediated Lysis

Meegan M. Gruber and David M. Kranz<sup>1</sup>

Department of Biochemistry, University of Illinois, Urbana, IL 61801

Running Title: Susceptibility of Breast Cancer to Lysis by CTL

<sup>1</sup> Address correspondence and reprint requests to Dr. David M. Kranz,  
Department of Biochemistry, University of Illinois, Urbana, IL 61801  
(217)244-2821, *fax*(217)244-5858.

## **Abstract**

Although bispecific antibodies to erbB-2 have been shown to redirect cell mediated cytotoxicity of erbB-2 positive tumor cells, there is evidence that some tumor cells are resistant to immune cytotoxic mechanisms. In this report, the susceptibility of erbB-2<sup>+</sup> breast cancer lines to lysis mediated by a bispecific antibody and the mouse cytotoxic T cell clone (CTL) 2C was evaluated. The bispecific single chain antibody (scFv<sub>2</sub>), 1B2/4420, which recognizes the T cell receptor on CTL 2C and the hapten fluorescein was used in order to eliminate the effect of erbB-2 density on recognition. Thus, covalent attachment of equivalent amounts of fluorescein to different tumor cells allowed this bispecific agent to be used to examine the intrinsic susceptibility of tumor cells to T cell mediated lysis. Among the panel of tumor lines tested, the absolute level of erbB-2 was not correlated with susceptibility. However, the erbB-2<sup>+</sup> cell line SKBR-3 was found to be highly resistant to lysis by CTL 2C. This observation prompted us to screen for agents that might increase the susceptibility of SKBR-3 to lysis. A combination of IFN- $\gamma$  and TNF- $\alpha$ , but not either agent alone, was effective at increasing susceptibility. This cytokine induced effect was not directly correlated with increased levels of ICAM-1 or Fas, and cytokine treatment did not alter the surface expression of erbB-2. These results indicate that some erbB-2<sup>+</sup> tumors may be resistant to the lytic mechanisms of cytotoxic T lymphocytes. Strategies that lead to localized expression of particular cytokine combinations, such as IFN- $\gamma$  and TNF- $\alpha$ , have potential in reducing this resistance.

**Key Words:** Bispecific Antibodies, erbB-2, Cytokines, IFN- $\gamma$ , TNF- $\alpha$

## Introduction

Human breast cancers that overexpress the oncogene product erbB-2 are usually aggressive tumors that lead to poor overall prognosis. Perhaps related to this observation are the results of several reports that suggest erbB-2 expression is correlated with resistance to lysis by TNF- $\alpha$  or lymphokine activated killer cells [9, 13, 20].

The presence of erbB-2 on the cell surface has provided a potential target for the elimination of these tumor cells using various antibody agents (reviewed in [4]). Bispecific antibodies have been used to effectively target some erbB-2<sup>+</sup> breast cancer cell lines for lysis by cytotoxic T lymphocytes. However, it is not clear if tumor cell lines which overexpress erbB-2 are equally susceptible to CTL-mediated lysis. If they are not, resistance may lead to difficulty in eliminating such tumors using immunotherapeutic strategies that involve bispecific antibodies to erbB-2 and the T cell receptor or that involve immunogenic peptides that elicit CTL activity.

In this study, the bispecific single chain antibody (scFv<sub>2</sub>), 1B2/4420, was used to evaluate the susceptibility of different erbB-2<sup>+</sup> breast cancer cell lines to cytotoxic T cell mediated lysis, and to screen agents which may increase susceptibility. The scFv<sub>2</sub> 1B2/4420 contains the V<sub>H</sub> and V<sub>L</sub> regions of an anti-T cell receptor antibody, 1B2, and the V<sub>H</sub> and V<sub>L</sub> regions of an anti-fluorescein antibody, 4420 [11]. Covalent attachment of equivalent amounts of fluorescein to different tumor cells allowed this bispecific agent to be used in examining the intrinsic susceptibility of the tumor cells to cytotoxic T cell mediated lysis. Using this approach, the erbB-2<sup>+</sup> cell line SKBR-3 was found to be highly resistant to lysis by the cytotoxic T cell clone 2C. This is in contrast to several other human and mouse tumor cell lines that were efficiently lysed, even when the antigen fluorescein was present at lower densities. The erbB-2<sup>+</sup> cell lines SKBR-3,

MCF-7, T-47D, MDA-MB-453, MDA-MB-468, SKOV-3, and BT-474 were found to be susceptible to CTL lysis to varying degrees. Susceptibility was not absolutely correlated with the relative levels of erbB-2 expressed on each cell line.

There is evidence that the cytokines TNF- $\alpha$  and/or IFN- $\gamma$  can increase susceptibility of some tumor cell lines to lymphokine activated killer cells and tumor infiltrating lymphocytes [1, 8, 26]. In addition, there has been speculation that in vivo administration of IFN- $\gamma$  and TNF- $\alpha$  may increase tumor susceptibility to CD8<sup>+</sup> TIL[2]. To determine whether these cytokines could increase susceptibility of erbB-2<sup>+</sup> tumors to CTL lysis, cytokine treated erbB-2<sup>+</sup> lines were labeled with FITC and assayed for lysis by the scFv2 and CTL clone 2C. A combination of IFN- $\gamma$  and TNF- $\alpha$ , but not either cytokine alone, was effective in increasing susceptibility to lysis (e.g. cytolysis of SKBR-3 increased on average from <5% to >30%). This combination was also able to increase the susceptibility of MCF-7, T-47D, and MDA-MB-361, but not MDA-MB-453, BT-474, or SKOV-3. The increase in susceptibility to lysis required incubation of tumor cells with the cytokines for 24 to 48 hours.

IFN- $\gamma$  and TNF- $\alpha$  may increase tumor cell susceptibility through a variety of mechanisms. Reports suggest that IFN- $\gamma$  and TNF- $\alpha$  decrease erbB-2 levels, rendering cells susceptible to immune cytotoxic mechanisms [19]. IFN- $\gamma$  and/or TNF- $\alpha$  have also been reported to increase cell surface densities of adhesion molecules [1, 21], MHC class I [14] and II [23], and tumor antigen [16]. Another possible mechanism of cytokine action was an increase in Fas, which could lead to enhanced interaction with Fas ligand (FasL) on the CTL [22], (reviewed in [15]). To examine several of these possibilities, the levels of erbB-2, ICAM-1, and Fas were examined after treatment with IFN- $\gamma$  and TNF- $\alpha$ , alone or in combination. Treatment with IFN- $\gamma$  alone led to an increase in ICAM-1 and Fas,



but not erbB-2. However IFN- $\gamma$  alone did not result in an increased susceptibility to lysis. We conclude that some other post-binding mechanism must account for the effects of the combination of IFN- $\gamma$  and TNF- $\alpha$ .

### **Materials and Methods**

**Cell Lines.** The BALB.B derived CTL clone 2C, which recognizes the alloantigen L<sup>d</sup> [17], was maintained in RPMI media (RPMI 1640, 5mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 $\mu$ M 2-mercaptoethanol, penicillin, streptomycin), and 10% supernatant from Con A-stimulated rat spleen cells as previously described [17]. 2C cells were stimulated approximately every week with mitomycin C-treated BALB/c spleen cells. Daudi, a human lymphoma that expresses class II HLA molecules, was maintained in RPMI media. Human breast and ovarian carcinomas were obtained from the ATCC (Rockville, MD). SKBR-3, and SKOV-3 were maintained in McCoy's 5A medium containing supplement (5mM HEPES, 10% fetal bovine serum, 1.3 mM L-glutamine, 50 $\mu$ M 2-mercaptoethanol, penicillin, streptomycin). T-47D and BT-474 were maintained in RPMI medium with 10 $\mu$ g/ml bovine insulin (GIBCO, Grand Island, NY). MDA-MB-453 and MDA-MB-468 were maintained in Leibovitz's L-15 medium containing supplement. MCF-7 was maintained in minimum essential medium with non-essential amino acids, 1mM sodium pyruvate, 10 $\mu$ g/ml bovine insulin, and supplement. MDA-MB-361 was maintained in DMEM/F12 with supplement. 900F4 hybridoma, kindly provided by P. Natali, was maintained in RPMI medium. (All media was obtained from GIBCO).

**Monoclonal Antibodies.** 900F4 is a BALB/c derived IgG1 monoclonal antibody that is specific for erbB-2 transmembrane glycoprotein[7]. 900F4 was purified from ascites fluid by ammonium sulfate precipitation followed by DEAE column chromatography. Anti-human ICAM (CD54) antibody was obtained

from PharMingen (SanDiego, CA). Anti-human Fas (CD95) monoclonal antibody UB2 was obtained from Kamiya Biomedical Co. (Thousand Oaks, CA). The single chain bispecific antibody (scFv2) contains the V<sub>H</sub> and V<sub>L</sub> regions of anti-TCR antibody 1B2 and the V<sub>H</sub> and V<sub>L</sub> regions of anti-fluorescein antibody 4420. The scFv2 construction was expressed, as previously described [11], using a hybrid O<sub>L</sub>/P<sub>R</sub>  $\lambda$  phage promoter in an *E.coli* strain that contains a temperature sensitive C1857 repressor gene. Induction, solubilization, and purification were performed as previously described[11]. Briefly, small scale cultures or fermentations were grown to an A<sub>600</sub> of 1.0 at 30°C, at which point recombinant protein expression was induced by temperature shift to 42°C for 1 hr. Cells were disrupted by passage through a microfluidizer, centrifuged at low speed to remove unlysed cells and the supernatant centrifuged to pellet inclusion bodies. Inclusion bodies were solubilized for 12-24 hours in 6M guanidine hydrochloride (10 ml guanidine per inclusion body pellet equivalent obtained from 1 liter of culture) then dialyzed against 0.1M Tris, pH 8.0, 2mM EDTA, pH 8.0, 0.4M arginine (TEA buffer) at 4°C. Fluorescein-specific scFv2 were isolated by affinity chromatography on Sepharose 6B conjugated to 5-aminofluorescein. The anti-fluorescein scFv2 antibodies were eluted with 50mM NaCO<sub>3</sub>, pH 11.5. All columns were equilibrated in 10mM Tris, pH 8.0. Fractions were dialyzed against 10mM Tris, or PBS pH 8.0 at 4°C.

**Cytokines.** Recombinant human IFN- $\gamma$  and TNF- $\alpha$  were obtained from R&D Systems (Minneapolis, MN). IFN- $\gamma$  was diluted to 10<sup>6</sup> units/ml in sterile filtered 10mM acetic acid, 0.1%BSA. TNF- $\alpha$  was diluted to 10<sup>6</sup> units/ml in sterile filtered PBS, 0.1%BSA. Aliquots were stored at -80°C.

**Cytotoxicity Assays.** Target cells were incubated for various times with different concentrations of IFN- $\gamma$ , TNF- $\alpha$ , or both prior to the cytotoxicity assay. Target cells were harvested by trypsinization, washed with RPMI medium, and

incubated with 50 $\mu$ l of  $^{51}\text{Cr}$  (2.5mCi/ml) for 1 hr at 37°C. After washing with PBS, cells were incubated in 1 ml PBS, pH 7.5 containing 100  $\mu$ M fluorescein isothiocyanate (Molecular Probes, Eugene, OR) for 15 min at 37°C. Target cells were washed in RPMI media and added to round bottom wells of a 96-well plate at  $2 \times 10^4$  cells/well. CTL clone 2C was washed with RPMI media prior to addition to wells. IFN- $\gamma$ , TNF- $\alpha$ , and scFv2 were added directly to the appropriate wells in the cytotoxicity assay. All assays were incubated for 4 hrs at 37°C in a 5% CO<sub>2</sub> incubator. Supernatants were removed and monitored with a  $\gamma$ -counter. Specific  $^{51}\text{Cr}$ -release was determined as follows: %Specific  $^{51}\text{Cr}$  release = ((experimental counts - spontaneous counts)/(maximal counts - spontaneous counts)) x 100.

**Flow Cytometry.** Cells were harvested by trypsinization, washed, and resuspended in RPMI media.  $5 \times 10^5$  cells were incubated with anti-ICAM antibody(anti-CD54) , anti-erbB-2 antibody 900F4, or RPMI media on ice for 30 min. The cells were washed in RPMI media and incubated with FITC labeled goat anti-mouse IgG (Kirkegaard and Perry, CITY ST) for 30 min. on ice. FITC labeled anti-Fas antibody UB2 was added to  $5 \times 10^5$  cells and incubated on ice for 30 min. For cells labeled directly with FITC,  $5 \times 10^5$  cells were incubated with 100 $\mu$ M FITC in PBS, pH 7.3 for 15 min. at 37°C. All cells were washed twice in RPMI media, resuspended in PBS, and analyzed on a Coulter EPICS at the University of Illinois Flow Cytometry Facility.

## Results

**Intrinsic susceptibility of human tumor lines to CTL lysis.** To examine the extent of susceptibility to lysis by CTLs, a panel of human tumor lines was assayed for lysis mediated by a bispecific antibody and the CTL clone 2C. To minimize the effects of recognition on susceptibility, target cells were labeled with fluorescein at approximately equivalent levels (Table 3 and data not

shown) and assayed for lysis in the presence of an anti-TCR/ anti-fluorescein bispecific antibody. As expected, the mouse L<sup>d</sup> alloreactive CTL 2C was unable to lyse FITC labeled human target cells in the absence of bispecific antibody (data not shown). Addition of the bispecific antibody resulted in CTL-mediated lysis of most of the target lines (Table 1). A wide range of susceptibilities, from 6% to 56% at an E:T ratio of 10:1 was observed for the panel of tumor lines. Although absolute cytotoxicity values varied between experiments (probably due to variations in killing efficiency of the CTL clone 2C; unpublished observation), the relative susceptibilities of different lines was consistent. The human lymphoma Daudi, a relatively susceptible cell line, was used in each assay for comparison.

Reports have suggested that erbB-2 overexpression is correlated with increased resistance to cellular cytotoxic mechanisms [9, 20]. To determine if susceptibility to CTL lysis was correlated with erbB-2 expression, erbB-2 levels were examined with the anti-erbB-2 antibody 900F4 by flow cytometry. Although the most resistant line SKBR-3 also expressed the highest level of erbB-2, there was not a strict correlation between erbB-2 levels and susceptibility (Figure 1).

**IFN- $\gamma$  and TNF- $\alpha$  increase target cell susceptibility.** To determine whether susceptibility to CTL lysis could be increased by treatment with IFN- $\gamma$  and TNF- $\alpha$ , the relatively resistant line SKBR-3 was incubated for 48 hours with increasing doses of the cytokines alone or in combination. After coupling of fluorescein, cells were assayed with the scFv2 and CTL 2C. Figure 2A illustrates the dose dependent increase in susceptibility of SKBR-3 after treatment with both IFN- $\gamma$  and TNF- $\alpha$ . Incubation with 1000U each of IFN- $\gamma$  and TNF- $\alpha$  resulted in maximal increase in susceptibility. This increase was not

seen with either IFN- $\gamma$  or TNF- $\alpha$  alone (Figure 2A) and it was not dependent on incubation of CTL 2C with cytokines (data not shown).

The kinetics of increased susceptibility were examined using 1000U each of IFN- $\gamma$  and TNF- $\alpha$ . SKBR-3 was assayed for susceptibility to CTL-mediated lysis after incubation with the cytokines for 10, 24, and 48 hours. Figure 2B shows that the maximal increase in susceptibility occurred after 24 hours.

To investigate cytokine induced increase in susceptibility in other erbB-2<sup>+</sup> tumor lines, MCF-7, MDA-MB-453, MDA-MB-468, T-47-D, SKOV-3, BT-474, and MDA-MB-361 were each incubated with 1000U of IFN- $\gamma$  and TNF- $\alpha$  for 48 hours. <sup>51</sup>Cr release of fluorescein labeled targets with scFv2 and CTL 2C resulted in cytokine induced increases in lysis for MCF-7, T-47-D, and MDA-MB-361, but no increase for MDA-MB-453, MDA-MB-468, SKOV-3, and BT-474 (Table 2). The latter tumor lines exhibited the most susceptibility without treatment. Thus, the cytolytic mechanism induced by cytokine treatment in the more resistant lines may already have been operative in the susceptible lines.

**Cytokine induced changes in erbB-2, ICAM-1 and Fas are not correlated with increased susceptibility.** To begin to study the mechanism responsible for the cytokine induced sensitivity to lysis, we examined several cell surface molecules known to be involved in effector/target cell interactions. Since cytokine induced susceptibility to CTL lysis was seen in some tumor lines but not in others, it was possible to determine relationships between cytokine induced effects on target cells and increased susceptibility. As discussed earlier, the level of overexpression of erbB-2 did not correlate with intrinsic susceptibility to CTL lysis. There are, however, reports that indicate some cytokines may decrease levels of erbB-2 rendering cells more susceptible to cytokine induced effects[19]. To determine whether a cytokine induced

decrease in erbB-2 was correlated with increased susceptibility, erbB-2 levels on IFN- $\gamma$  and TNF- $\alpha$  treated cells were examined by flow cytometry using anti-erbB-2 antibody 900F4. No significant decrease was seen in any of the lines, indicating that a change in erbB-2 expression was not a likely mechanism of this cytokine action (Table 3).

Increases in ICAM-1 after cytokine treatment have been implicated in IFN- $\gamma$  and TNF- $\alpha$  induced increases in susceptibility to cellular cytotoxic mechanisms[21]. To determine if IFN- $\gamma$  and TNF- $\alpha$  affect ICAM-1 levels, and if the effect is correlated with increased susceptibility, erbB-2<sup>+</sup> cell lines were incubated with 1000U of cytokines for 48 hours, and examined with an anti-ICAM-1 antibody by flow cytometry. As previously reported[1, 21], incubation with cytokines increased ICAM-1 levels in all cell lines tested (Table 3). However, the increase was not directly correlated with increased susceptibility since ICAM-1 levels were also raised for cells treated with IFN- $\gamma$  alone (Table 4). As indicated above, IFN- $\gamma$  treatment alone did not increase susceptibility to lysis.

Interaction between Fas on target cells and the FasL on T cells can lead to apoptosis of the target [22]. To investigate the possibility of cytokine induced Fas elevation on treated targets, cells were incubated with IFN- $\gamma$  alone, TNF- $\alpha$  alone, or a combination of both for 48 hours. Flow cytometric analysis with FITC-labeled anti-Fas antibody UB2 showed similar results as were obtained for ICAM-1 (Table 4). That is, although Fas levels were slightly elevated with IFN- $\gamma$  and TNF- $\alpha$ , they were also elevated with IFN- $\gamma$  alone. These results suggest that there exists a mechanism of action separate from ICAM-1 and Fas level elevation, through which IFN- $\gamma$  and TNF- $\alpha$  together increase target cell susceptibility.

## Discussion

The breast cancer cell lines SKBR-3 and MCF-7 were found to be highly resistant to lysis mediated by CTL clone 2C while MDA-MB-453, MDA-MB-468, MDA-MB-361, T-47-D, and SKOV-3 were more susceptible to lysis. Differences in susceptibility were not directly correlated with erbB-2 or ICAM-1 levels on the tumor cell surface. Previous studies have demonstrated a cytokine induced increase in susceptibility of tumor cells to TIL and LAK cell lysis[8, 20, 26]. In this study, we examined if cytokines could similarly increase susceptibility of erbB-2<sup>+</sup> tumors to CTL lysis. A combination of IFN- $\gamma$  and TNF- $\alpha$  was able to increase the susceptibility of erbB-2<sup>+</sup> tumors to bispecific antibody mediated CTL lysis. In contrast to results by Lichtenstein et al.[20], treatment with cytokines did not result in a significant effect on the cell surface level of erbB-2.

Previous studies have examined susceptibility of tumor cells to cytotoxicity in terms of target cell resistance to effector cell binding as well as resistance to post-binding cytolytic mechanisms [9, 26]. Resistance to effector cell binding can involve the down regulation of surface molecules important for adhesion and/or recognition by the effector cell. Resistance to post binding mechanisms are less well characterized but presumably it involves either the pathway that leads to apoptosis (Fas/FasL) or the pathway that leads to membrane destruction by cytolytic granules and perforin.

To examine if enhanced effector cell binding might be a possible mechanism of the cytokine induced increase in susceptibility to CTL lysis, surface levels of several key molecules were measured after cytokine treatment. Although IFN- $\gamma$  has been reported to increase MHC levels and thus antigen presentation on target cells, the bispecific antibody system used here minimizes the role of MHC/peptide in CTL activation. However, the role of ICAM-1 in antibody redirected cytotoxicity has been documented[1, 10, 18, 21]. To

determine the role of ICAM-1 expression in resistance to CTL lysis and cytokine induced susceptibility, surface expression of ICAM-1 was measured before and after cytokine treatment. Susceptibility of untreated cells was not correlated with ICAM-1 levels. In contrast to previous reports[9], ICAM-1 levels were also not correlated with overexpression of erbB-2 and were similar to levels expressed on erbB-2<sup>-</sup> cells. Although there was a significant increase in ICAM-1 density after treatment with IFN- $\gamma$  and TNF- $\alpha$ , this increase was similar to that seen with IFN- $\gamma$  alone, and therefore was not sufficient to explain the synergistic mechanism necessary to effect increased susceptibility to lysis.

Similar results were seen for Fas antigen on the surface of the erbB-2<sup>+</sup> cells. Thus, CTL induced apoptosis as a result of enhanced interactions between Fas and FasL did not appear to account for the increase in lysis (ie. surface levels of Fas increased after treatment with IFN- $\gamma$  alone, as well as with both IFN- $\gamma$  and TNF- $\alpha$ ).

Previous studies examining the susceptibility of erbB-2<sup>+</sup> cells to cytolytic post-binding mechanisms have demonstrated resistance to lysis even in the presence of the lectin Concanavalin A, as well as resistance to lytic perforin granules[9]. In apparent contradiction to the report of Fady *et al.* [9] and the results presented here, several studies have shown that erbB-2<sup>+</sup> SKBR-3 could be lysed by CTLs [24, 25, 29]. At this time the explanation for these discrepancies is not known. However, one difference between our study and the others, is the source of effector CTL (mouse CTL clone 2C versus polyclonal human CTL lines). If SKBR-3 is in fact resistant to lysis by the perforin pathway, then the Fas/FasL pathway may be the major mechanism by which human CTL act on SKBR. Accordingly, the mouse CTL clone 2C may not have been able to mediate lysis via this pathway, even though Fas is thought to lack species specificity [27] and CTL 2C has been shown to mediate calcium independent



lysis that is characteristic of the Fas pathway (William Soo Hoo and David M. Kranz, unpublished results). Treatment with the combination of IFN- $\gamma$  and TNF $\alpha$  could presumably have an affect on either the perforin or the Fas/FasL pathway. We are not aware of studies which have directly addressed either of these possibilities.

Reports on the synergism of IFN- $\gamma$  and TNF- $\alpha$  have frequently studied the direct action of these cytokines on effector cell activation[5, 12]. Discussion of cytokine induced effects on *in vivo* tumor regression have also focused largely on the increase in activity of cytolytic cells [3, 6] or the direct action of the cytokines on tumor cell proliferation [6, 28]. Our results suggest that the tumoricidal effects of IFN- $\gamma$  and TNF- $\alpha$  treatment may, in the case of some tumors, be due not only to enhancement of the cytolytic ability of effector cells but also to increased susceptibility of the tumor to the cytolytic machinery.

### **Acknowledgements**

We greatly acknowledge Dr. Pier Natali for providing the hybridoma 900F4. This work was supported by a grant (DAMD-17-94-J-4347) from the U.S. Army. M.M.G was generously supported by a research scholarship from the Carle Cancer Center.

## References

1. Azuma A, Yagita H, Matsuda H, Okumura K, Niitani H (1992) Induction of intercellular adhesion molecule 1 on small cell lung carcinoma cell lines by  $\gamma$ -interferon enhances spontaneous and bispecific anti-CD3 x antitumor antibody-directed lymphokine killer cell cytotoxicity. *Cancer Res* 52: 4890
2. Barth RJ, Mule JJ, Spiess PJ, Rosenberg SA (1991) Interferon  $\gamma$  and tumor necrosis factor have a role in tumor regressions mediated by murine CD8+ tumor-infiltrating lymphocytes. *J Exp Med* 173: 647
3. Brouckaert PGG, Leroux-Roels GG., Guisez Y, Tavernier J, and Fiers W (1986) In vivo anti-tumor activity of recombinant human and murine TNF, alone and in combination with murine IFN- $\gamma$ , on a syngeneic murine melanoma. *Int J Canc* 38: 763
4. Carter P, Rodriguez ML, Lewis GD, Figari I, Shalaby MR (1994) Towards an immunotherapy for p185HER2 overexpressing Tumors. In: R. L. Ceriani (ed.) *Antigen and antibody molecular engineering in breast cancer diagnosis and treatment*. Plenum, New York, p83.
5. Chen L, Suzuki Y, Wheelock EF (1987) Interferon- $\gamma$  synergizes with tumor necrosis factor and with interleukin 1 and requires the presence of both monokines to induce antitumor cytotoxic activity in macrophages. *J Immunol* 139: 4096
6. Demanet C, Brissinck J, Leo O, Moser M, Thielemans K (1994) Role of T-cell subsets in the bispecific antibody (anti-idiotypic x anti-CD3) treatment of the BCL1 lymphoma. *Cancer Res* 54: 2973
7. Digiesi G, Giacomini P, Fraioli R, Mariani M, Nicotra MR, Segatto O, Natali PG (1992) Production and characterization of murine mAbs to the extracellular domain of human Neu oncogene product GP185<sup>HER2</sup>. *Hybridoma* 11: 519

8. Fady C, Gardner AM, Gera JF, Lichtenstein A (1992) Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells: selective effects on HER2/*neu*-overexpressing cells. *Cancer Res* 52: 764
9. Fady C, Gardner A, Gera JF, Lichtenstein A (1993) Resistance of HER2/*neu*-overexpressing tumor targets to lymphokine-activated-killer cell-mediated lysis: evidence for deficiency of binding and post-binding events. *Cancer Immunol Immunother* 36: 307
10. Ferrini S, Sforzini S, Camniaggi A, Poggi A, Meazza R, Canevari S, Colnaghi MI, Moretta L (1994) The LFA-1/ICAM cell adhesion pathway is involved in tumor-cell lysis mediated by bispecific monoclonal-antibody-targeted T lymphocytes. *Int J Cancer* 56: 846
11. Gruber M, Schodin B, Wilson E, Kranz DM (1994) Efficient Tumor Cell Lysis Mediated by a Bispecific Single-Chain Antibody Expressed in *E. coli*. *J Immunol* 152: 5368
12. Hori K, Ehrke MJ, Mace K, Mihich E (1987) Effect of recombinant tumor necrosis factor on tumoricidal activation of murine macrophages: synergism between Tumor necrosis factor and  $\gamma$ -interferon. *Cancer Res* 47:5868
13. Hudziak RM, Lewis GD, Shalaby RM, Eessalu TE, Aggarwal BB, Ullrich A, Shepard HM (1988) Amplified expression of the HER2/ERBB2 oncogene induces resistance to tumor necrosis factor  $\alpha$  in NIH 3T3 cells. *Proc Natl Acad Sci, USA* 85: 5102
14. Imai K, Ah-Kau N, Glassy MC, Ferrone S (1981) Differential effect of interferon on the expression of tumor-associated antigens and histocompatibility antigens on human melanoma cells: relationship to susceptibility to immune lysis mediated by monoclonal antibodies. *J Immunol* 127: 505

15. Kagi D, Ledermann B, Burki K, Zinkernagel RM, Hengartner H (1995) Lymphocyte-mediated cytotoxicity in vitro and in vivo: mechanisms and significance. *Immunol Rev* 146: 95
16. Klyczek KK, Murasko DM, Blank KJ (1987) Interferon- $\gamma$ , interferon- $\alpha/\beta$ , and tumor necrosis factor differentially affect major histocompatibility complex Class I expression in murine leukemia virus-induced tumor cell lines. *J Immunol* 139: 2641
17. Kranz DM, Sherman DH, Sitkovsky MV, Pasternack MS, Eisen HN (1984) Immunoprecipitation of cell surface structure of cloned cytotoxic T lymphocytes by clone-specific antisera. *Proc Natl Acad Sci, USA* 81: 573
18. Kranz DM, Tonegawa S, Eisen HN (1984) Attachment of an anti-receptor antibody to non-target cells renders them susceptible to lysis by a clone of cytotoxic T lymphocytes. *Proc Natl Acad Sci, USA* 81: 7922
19. Kumar R, Mendelsohn J (1994) Reduced expression of c-erbB-2 gene product in human mammary carcinoma SK-BR-3 cells treated with interferon- $\gamma$  and tumor necrosis factor- $\alpha$ . *Anticancer Res* 14: 1001
20. Lichtenstein A, Berenson J, Gera JF, Waldburger K, Martinez-Maza O, Berek JS (1990) Resistance of human ovarian cancer to tumor necrosis factor and lymphokine-activated killer cells: correlation with expression of HER2/neu oncogenes. *Cancer Res* 50: 7364
21. Ramsey PS, Dean PA, Tasimowicz-Alpini JH, Donohue JH, Nelson H (1993) Interferon- $\gamma$ -induced intercellular adhesion molecule-1: expression on human tumor cells enhances bifunctional antibody-mediated lysis through a lymphocyte function-associated antigen-1 dependent mechanism. *Cancer Res* 53: 4652
22. Rouvier E, Luciani MF, Golstein P (1993) Fas involvement in  $Ca^{2+}$  independent T cell-mediated cytotoxicity. *J Exp Med* 177: 195

23. Ruggiero P, Bender JR, Engleman EG (1987.) Lymphocyte subsets differentially induce class II human leukocyte antigens on allogeneic microvascular endothelial cells. *J Immunol* 139: 2585.
24. Shalaby MR, Shepard HM, Presta L, Rodrigues ML, Beverly PCL, Feldman M, Carter P (1992) Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene. *J Exp Med* 175: 217
25. Shalaby MR, Carter P, Maneval D, Giltinan D, Kotts C (1995) Bispecific HER2xCD3 antibodies enhance T-cell cytotoxicity *in vitro* and localize to HER2-overexpressing xenografts in nude mice. *Clin Immunol Immunopath* 74: 185
26. Stotter H, Wiebke EA, Tomita S, Beldegrun A, Topalian S, Rosenberg SA, Lotze MT (1989) Cytokines alter target cell susceptibility to lysis. II. Evaluation of tumor infiltrating lymphocytes. *J Immunol* 153: 1767
27. Tanaka M, Suda T, Takahashi T, Nagata S (1995) Expression of the functional soluble form of human Fas ligand in activated lymphocytes. *EMBO J* 14: 1129
28. Tuttle TM, McCrady CW, Inge TH, Salour M, Bear HD (1993)  $\gamma$ -interferon plays a key role in T-cell-induced tumor regression. *Cancer Res* 53: 833
29. Zhu Z, Zapata G, Shalaby R, Snedecor B, Chen H, Carter P (1996) High level secretion of a humanized bispecific diabody from *Escherichia coli*. *Biotech* 14: 192

Table 1. Intrinsic Susceptibility of Human Breast Cancer Lines to Bispecific Antibody Mediated CTL Lysis.

Experiment	scFv2 μg/ml	E:T	% Specific <sup>51</sup> Cr Release								
			Daudi	SKBR -3	MDA- MB- 453	MCF- 7	MDA- MB- 468	T-47- D	SKOV -3	BT- 474	MDA- MB- 361
1	25	10:1	54	8							
2	50	6:1	82		32						
3	50	30:1	64		27						
4	18	6:1	24	1	4	3	14				
5	50	10:1	54	8	36	6	42	20			
6	50	10:1	56	18	45	24		36	38	53	38

A standard 4 hr <sup>51</sup>Cr release was performed on cells incubated with 125μCi <sup>51</sup>Cr for 1 hr at 37°C. Cells were washed and incubated in 100μM FITC in PBS, pH7.3 for 15 min at 37°C, washed, and plated at 2x10<sup>4</sup> cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO<sub>2</sub> incubator. Supernatants were removed and monitored in a γ-counter. Percent specific <sup>51</sup>Cr release was calculated as [(experimental cpm-spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Table 2. Effect of Treatment with IFN- $\gamma$  and TNF- $\alpha$  on Susceptibility of Cell Lines to CTL Lysis.

	Effector:Target	Cell Line	% Specific <sup>51</sup> Cr Release	
			untreated	treated
Experiment 1	10:1	Daudi	50	-
		SK-BR-3	1	29
Experiment 2	10:1	Daudi	51	
		SK-BR-3	3	46
Experiment 3	10:1	Daudi	54	56
		SK-BR-3	8	30
		MCF-7	6	15
		MDA-MB-453	36	35
		MDA-MB-468	42	39
		T-47-D	20	46
Experiment 4	10:1	Daudi	56	63
		SK-BR-3	18	28
		MCF-7	24	35
		MDA-MB-453	45	40
		T-47-D	36	54
		SKOV-3	38	38
		BT-474	53	39
		MDA-MB-361	38	45

Cells were incubated for 48 hours in 1000U/ml IFN- $\gamma$  and TNF- $\alpha$ , or RPMI medium in a 5% CO<sub>2</sub> incubator at 37°C. Cells were harvested by trypsinization, washed in RPMI media and incubated with 125 $\mu$ Ci <sup>51</sup>Cr for 1 hr at 37°C. Cells were washed and incubated in 100mM FITC in PBS, pH7.3 for 15 min at 37°C, and plated at 2x10<sup>4</sup> cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO<sub>2</sub> incubator. Supernatants were removed and monitored in a  $\gamma$ -counter. %Specific <sup>51</sup>Cr Release=[(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.

Table 3. Effect of IFN- $\gamma$  and TNF- $\alpha$  on ICAM-1 and erbB-2 levels.

Cell Line	IFN- $\gamma$ /TNF- $\alpha$	erbB-2 (MFU)	ICAM-1 (MFU)	FITC (MFU)	% Lysis
SKBR	-	188	115	207	8
SKBR	+	185	189	198	30
MCF-7	-	84	135	204	6
MCF-7	+	79	194	196	15
MDA-MB-453	-	142	109	179	36
MDA-MB-453	+	129	180	182	35
T-47-D	-	96	91	185	20
T-47-D	+	97	172	185	46
Daudi	-	11	78	156	54
Daudi	+	8	94	157	56

Cells were incubated for 48 hours in 1000U/ml IFN- $\gamma$  and TNF- $\alpha$  or RPMI media in a 5% CO<sub>2</sub> incubator at 37°C. Cells were harvested by trypsinization, washed in RPMI media and split for <sup>51</sup>Cr release and flow cytometry. For flow cytometric analysis, 5x10<sup>5</sup> cells were incubated with anti-ICAM-1, anti-erbB-2 (900F4), or RPMI media 30 min on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. Alternatively, cells were directly labeled with 100 $\mu$ M FITC in PBS, pH7.3 for 15 min at 37°C. All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. Data shown in mean fluorescence units (MFU). A standard 4 hr <sup>51</sup>Cr release was performed on cells incubated with 125 $\mu$ Ci <sup>51</sup>Cr for 1 hr at 37°C. Cells were washed and incubated in 100 $\mu$ M FITC in PBS, pH7.3 for 15 min at 37°C, and plated at 2x10<sup>4</sup> cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at 37°C in a 5% CO<sub>2</sub> incubator. Supernatants were removed and monitored in a  $\gamma$ -counter. %lysis=[(experimental cpm - spontaneous cpm)/(maximal cpm - spontaneous cpm)]x100.



Table 4. Effect of IFN- $\gamma$  and/or TNF- $\alpha$  on ICAM-1 and Fas levels.

Cell Line	Treatment	ICAM-1 (MFU)	Fas (MFU)
SKBR	-	117	57
SKBR	IFN- $\gamma$	273	84
SKBR	TNF- $\alpha$	235	63
SKBR	IFN- $\gamma$ /TNF- $\alpha$	287	86
MCF-7	-	129	48
MCF-7	IFN- $\gamma$	176	61
MCF-7	TNF- $\alpha$	146	52
MCF-7	IFN- $\gamma$ /TNF- $\alpha$	212	69

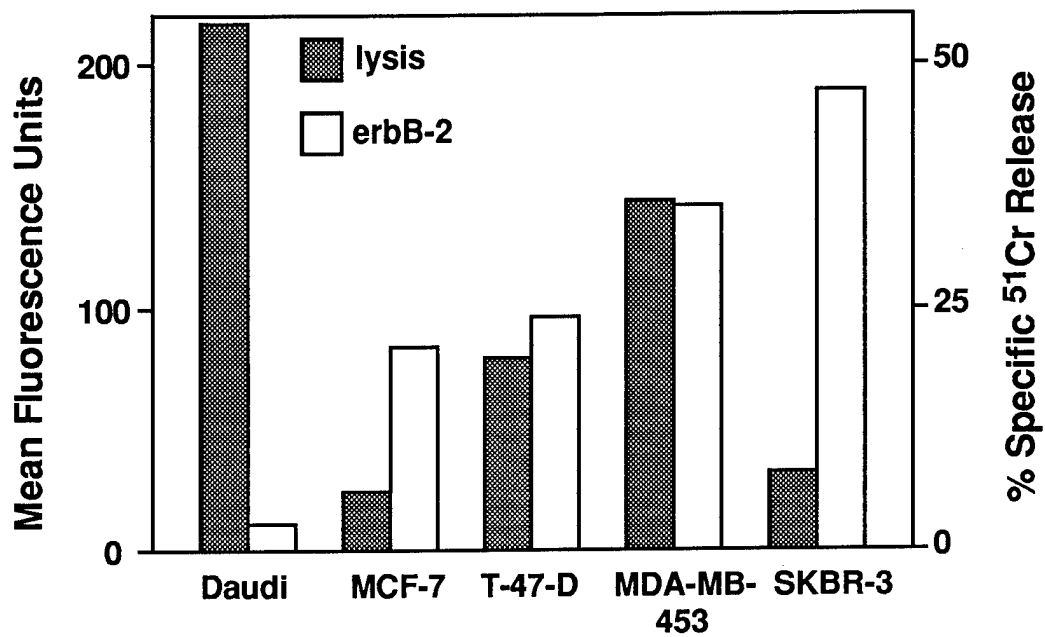
Cells were incubated for 48 hours in 1000U/ml IFN- $\gamma$ , TNF- $\alpha$  or RPMI media in a 5% CO<sub>2</sub> incubator at 37°C.  $5 \times 10^5$  cells were harvested by trypsinization, washed in RPMI media and incubated with anti-ICAM-1, anti-Fas, or RPMI media 30 min on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. Data shown in mean fluorescence units (MFU).

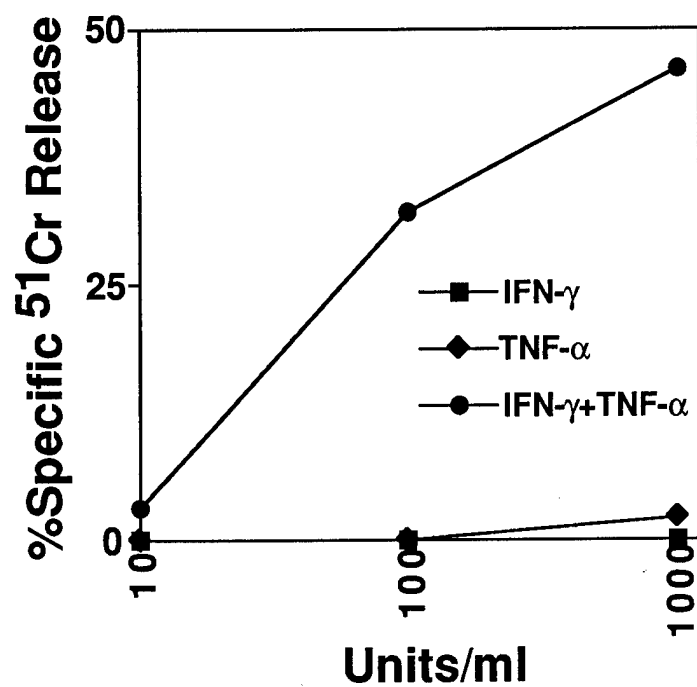
## Figure Legend

**Fig 1. Susceptibility of human breast cancer lines is not correlated to level of overexpression of erbB-2.** Cells were harvested by trypsinization, washed in RPMI media and split for  $^{51}\text{Cr}$  release and flow cytometry. For flow cytometric analysis,  $5 \times 10^5$  cells were incubated with anti-erbB-2 (900F4), or RPMI media 30 min. on ice. Cells were washed and labeled with FITC labeled goat anti-mouse IgG for 30 min on ice. Alternatively, cells were directly labeled with  $100 \mu\text{M}$  FITC in PBS, pH7.3 for 15 min at  $37^\circ\text{C}$ . All cells were washed twice, resuspended in PBS, and analyzed on a Coulter EPICS. A standard 4 hr  $^{51}\text{Cr}$  release was performed on cells incubated with  $125 \mu\text{Ci}$   $^{51}\text{Cr}$  for 1 hr at  $37^\circ\text{C}$ . Cells were washed and incubated in  $100 \mu\text{M}$  FITC in PBS, pH7.3 for 15 min at  $37^\circ\text{C}$ , and plated at  $2 \times 10^4$  cells per well. CTL 2C was added at an E:T ratio of 10:1 and incubated at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator. Supernatants were removed and monitored in a  $\gamma$ -counter. %Specific  $^{51}\text{Cr}$  Release =  $[(\text{experimental cpm} - \text{spontaneous cpm})/(\text{maximal cpm} - \text{spontaneous cpm})] \times 100$ .

**Fig 2. A. Susceptibility of SKBR-3 to CTL mediated cytotoxicity is increased by pretreatment with increasing doses of IFN- $\gamma$  or TNF- $\alpha$  but not by IFN- $\gamma$  or TNF- $\alpha$  alone.** SKBR-3 were incubated for 48 hours in 0, 10, 100, or 1000U/ml IFN- $\gamma$ , TNF- $\alpha$ , or a combination of both in a 5%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$ . Cells were harvested by treatment with trypsin-EDTA, washed with RPMI media and incubated with  $50 \mu\text{l}$  of  $^{51}\text{Cr}$  ( $2.5 \text{mCi/ml}$ ) for 1 hr at  $37^\circ\text{C}$ . Target cells were then incubated in  $1 \text{ml}$   $100 \mu\text{M}$  FITC for 15 min at  $37^\circ\text{C}$ , washed in RPMI media, and plated at  $2 \times 10^4$  cells/well. 2C cells and scFv2 were added directly to the CTL/target assay. All assays were performed at an E:T ratio of 10:1, incubated 4 hrs at  $37^\circ\text{C}$ , and supernatants removed and monitored by  $\gamma$ -counter. **B. Preincubation of SKBR-3 with 1000 units/ml**

each of IFN- $\gamma$  and TNF- $\alpha$  increases susceptibility to CTL mediated lysis in a time dependent manner. SKBR-3 were incubated in 1000U/ml IFN- $\gamma$  and TNF- $\alpha$  for 0, 10, 24, or 48 hours in 5%CO<sub>2</sub> incubator at 37°C. Cells were harvested by treatment with trypsin-EDTA washed with RPMI media and incubated with 50 $\mu$ l of <sup>51</sup>Cr (2.5mCi/ml) for 1 hr at 37°C. Target cells were then incubated in 1ml 100 $\mu$ M FITC for 15min at 37°C, washed in RPMI media, and plated at 2x10<sup>4</sup> cells/well. 2C cells and scFv<sub>2</sub> were added directly to the CTL/target assay. All assays were performed at an E:T ratio of 10:1, incubated 4 hrs at 37°C, and supernatants removed and monitored by  $\gamma$ -counter.



**A****B**